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COMPLEMENT FIXATION IN INFLUENZA *

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It was thought that it might be of interest to examine the serum of influenza patients for immune bodies that would bind complement in the presence of antigens made from organisms isolated in this disease. The results of cultures reported from different places varied so greatly that it was decided to obtain both serum and bacterial strains for antigens from several sources.

Convalescent serum was obtained from Michael Reese Hospital, Wesley Memorial Hospital and the University of Chicago, Chicago; from Camp Green, N. C.; from Camp Grant, Ill., and from the U. S. Naval Hospital, the U. S. Naval Medical School and the Hygienic Laboratory, Washington, D. C. The serum of four convalescent scarlet fever patients, of three convalescent diphtheria patients and from seven persons who had not had influenza, was used as controls.

Ten of the convalescent serums from Michael Reese Hospital were from moderately ill, uncomplicated cases of influenza, four from patients with influenza complicated by pneumonia. Of the latter, serum 3 came from a patient who had a very severe illness extending over 5 weeks. Other complicated cases were only moderately ill. In all cases, the serum was obtained from 3-7 seven days after the temperature had reached normal. The serum from Camp Green was obtained from a patient who several weeks before had a typical attack of influenza. Five serums from Wesley Memorial Hospital were from uncomplicated influenza and two from influenza and pneumonia. Details as to the nature of the influenzal attack were not obtained in the other cases.

As antigens, streptococci from blood cultures made in the Michael Reese Hospital were used. The streptococci were of two kinds:

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(1) A nonhemolytic, green-producing diplococcus; the colonies, which appeared in 24 hours, were moist, elevated, with slightly irregular margins, and produced no hemolysis during the 10 days in which they were observed; microscopically, it was a fair-sized, gram-positive diplococcus in pairs and chains. (2) A small gram-positive coccus in pairs and chains which after 24-48 hours appeared as minute green colonies and after 48-72 hours formed a very slight zone of almost complete hemolysis. Both varieties fermented levulose and dextrose, but not inulin nor mannite, and were not soluble in bile. Antigens were made from two strains of the first and four strains of the second. Other bacteria used for antigens were as follows: Influenza bacilli isolated from cases of influenza in Chicago and New York City; (1) atypical viridans streptococci isolated from cases of influenza by the late Capt. George Mathers at Camp Meade, Md.; similar cocci isolated by Dr. Ruth Tunnicliff from brains of persons dying with influenzal pneumonia in Chicago; pneumococci of different types; typical hemolytic streptococcus from a brain abscess; also *B. mucosus* and *M. catarrhalis*.

The serums were heated for 30 minutes at 56 C. and used undiluted with the exception of the specimens from the U. S. Naval Medical School, Washington, D. C., which contained a small amount of sodium citrate.

Unheated bacterial suspensions were used as antigens. In addition to the bacterial antigens two antigens were made from the sputum of patients severely ill with influenza; the sputum was ground with sterile sand and salt solution, and part of the suspension filtered through a Berkefeld filter; cultures of the filtrate were sterile; the other part was centrifugalized slowly to remove the larger particles, the supernatant fluid heated and used as antigen. In order to have sufficient serum for many antigens, only two antigen dilutions were used, $\frac{1}{4}$ and $\frac{1}{40}$ of the anticomplementary unit.

The antishoop rabbit system was employed in $\frac{1}{10}$ the volume of the original Wassermann test. Serum, antigen and complement (fresh guinea-pig serum, 2 units) were incubated at 37 C. for 1 hour when 2 units of previously titrated antishoop amboceptor and sheep corpuscles (5% suspension) were added, and the whole incubated for 1 hour. The customary antigen, serum and hemolytic control tests were made each time.



In some instances, especially when citrated serum was used, it was necessary to place the test in the icebox over night before a final reading could be made.

A negative Wassermann was obtained in the case of each serum used in the experiments.

The results which are given in tables 1 and 2 show that the antigens from Camp Meade and similar strains of streptococci of the viridans group were the only ones that fixed complement with any degree of constancy. One hundred forty-one serums of convalescent patients were tested with the Michael Reese atypical *Streptococcus viridans* (slight hemolysis) antigen. In 40% of the tests there was some degree of fixation. With the other Michael Reese atypical *Streptococcus viridans* (no hemolysis) there was fixation in 32% of 78 tests. The Camp Meade strain was used in 484 tests, 70% of which gave fixation. With the 176 tests made with the brain antigens, fixation occurred in 42%. Filtered sputum (52 tests) and washed sputum (19 tests) gave only 20 and 21% positive fixations, respectively. With various influenza antigens 240 tests were made with fixation in 20%. Of the 281 tests with pneumococcus antigen 15% fixed complement. Antigens from hemolytic streptococcus and from *B. mucosus* gave no fixation. Three serums gave partial inhibition of hemolysis with *M. catarrhalis*, when it was used as antigen. The scarlet fever serum fixed complement in five instances and the diphtheria serum in three. The seven normal serums used as controls gave uniformly negative results.

Serum was collected on several successive days from 12 patients. The collection of serum was started on the day the patient entered the hospital, which in most cases was early in the disease. These serums were tested with Camp Meade strain 2, which had given good fixation in previous tests, and also when the amount of serum permitted with influenza bacillus strain 101. The results (table 3) show that there was considerable fixation with the Camp Meade antigen, and that apparently little relation could be made out between the degree of fixation and the stage of the disease in which the serum had been obtained. In 12 cases only a very small amount of serum was collected; ten of the twelve specimens gave definite fixation with Camp Meade strain 2.

TABLE 2
PERCENTAGES OF FIXATION AS SHOWN BY TABLE 1

Antigens	No. of Tests with Influenza Serum	Fixation	No. of Tests with Scarlet Fever Serum	Fixation	No. of Tests with Diphtheria Serum	Fixation	No. of Tests with Normal Serum	Fixation
Atypical streptococci viridans group:								
Michael Reese Hospital (1).....	157	40%	8	0	6	0	28	0
Michael Reese Hospital (2).....	78	32%	8	0	6	0	14	0
Camp Meade (Captain Mathers)	484	70%	32	0	24	0	63	0
Brain (Dr. Tunnicliff).....	176	42%	20	5%	15	3%	35	0
Filtered sputum.....	52	20%	4	0	2	0	7	0
Washed sputum.....	19	21%	0	0	0	0	7	0
Influenza bacillus.....	253	20%	20	0	15	0	49	0
Pneumococcus.....	281	15%	24	0	18	0	42	0
Hemolytic streptococcus.....	36	8%	4	0	3	0	7	0
B. mucosus.....	21	0%	0	0	0	0	7	0
M. catarrhalis.....	33	15%	4	0	3	0	7	0

TABLE 3
TESTS FOR COMPLEMENT FIXATION WITH SERUMS COLLECTED ON DIFFERENT DAYS OF THE ATTACK

Case and Dates	Camp Meade Strain 2	Influenza Bacillus Strain 101	Case and Dates	Camp Meade Strain 2	Influenza Bacillus Strain 101
1 10/26	+++	0	9 11/11	0	0
10/27	+++	0	11/12	+	0
10/28	C	0	11/13	+	0
10/29	++	0	11/14	++	0
	++	0	10 11/15	++	0
2 10/26	0	0	11/16	+++	0
10/27	++	0	11/ 5	++	0
10/28	0	0	11/ 6	+++	0
10/29	++	0	11/10	++	0
3 10/25	++	0		+	0
10/26	++	0		++	0
10/27	++	0	12 11/ 7	++	0
10/28	0	0	11/ 8	+++++	0
10/29	++	0	11/10	++	0
	0	0	13 11/ 9	+++	0
4 10/16	0	0	14 11/ 9	++++	0
	++	0	15 11/ 9	++	0
5 10/15	0	0	16 11/ 9	+++	0
	++	0	17 11/ 9	++	0
6 10/13	0	0	18 11/25	0	0
10/14	0	0	19 11/25	0	0
7	0	0	20 10/29	++	0
	+++	0	21	++	0
8 10/15	0	0	22	++	0
11/ 4	0	0	23	+++++	0
11/ 7	0	0	24	+	0
11/ 8	0	0			
11/10	0	0			
	0	0			
	0	0			

SUMMARY

The outstanding feature of this work on complement fixation with influenza serum is the large number of positive results with certain strains of the viridans group of streptococci isolated from cases of influenza at Camp Meade and in Chicago. The evidence indicates that such organisms probably played an important part in the morbid process even in other places. Serum from influenza patients in several different places appears to have acquired similar new properties.

OBSERVATIONS ON THE BACTERIOLOGY OF INFLUENZA AND BRONCHOPNEUMONIA *

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This report is based on a study of cases admitted to St. Luke's hospital with a diagnosis of influenza or pneumonia between Oct. 1, 1918, and Jan. 15, 1919. The patients did not belong to any one group, but came from all parts of the city. Some were travellers who became sick enroute and were taken off the train at Chicago. Except for 14 soldiers, the patients were civilians.

BACTERIOLOGY OF THE SPUTUM

Specimens of sputum were obtained by asking the patients to cough and spit into a sterile bottle which was immediately taken to the laboratory. The sputum, when tenacious enough, was washed by gently shaking in three changes of sterile broth or salt solution. A direct smear of part of the washed sputum was stained by Gram's method, and part was plated on fresh human blood agar and incubated at 37 C. Anaerobic human blood agar plates, semicoagulated horse serum shake cultures, and cultures in broth with varying amounts of whole human blood, serum, or washed red cells were made in 20 cases. While a greater variety of organisms could be cultivated by these methods, it was found that the bacteria which predominated in direct smears of good specimens of sputum grew aerobically on human blood-agar plates.

Considerable difficulty was encountered in collecting sputum. Not every patient could cough up material even on persistent effort. Frequently specimens consisted of thin, clear, somewhat frothy mucus. Such sputum showed a variety of organisms, large squamous epithelial cells and few or no leukocytes. There was a tendency for the bacteria to group about the squamous epithelial cells, and large numbers of streptococci were often attached to them. These streptococci were usually green forming, but sometimes hemolytic. The other organisms often found in this type of sputum were spirilla, fusiform bacilli, staphylococci, micrococcus catarrhalis, gram-positive diphtheroid bacilli, and very minute gram-negative anaerobic cocci and bacilli. As this kind of sputum may not originate at the site of the primary infection, but comes largely from the mouth and throat, not much importance attaches to the results of cultures made with it.

Specimens of coughed-up sputum, which were tenacious, showed the least variety of organisms, and it was only from this type of sputum that anything like pure cultures was obtained. The specimens were produced after an attack of coughing, which cost the patient considerable effort. The cough, though initiated voluntarily, if it was to be productive, passed out of the patient's

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control, became spasmodic, sometimes ending in wrenching, and finally resulted in a clump of thick, very tenacious, clear or mucopurulent sputum.

Later in the disease when the patient was convalescent from pneumonia, the sputum was more abundant and less tenacious. A few cases had a persistent, productive cough until the time of discharge. This was ascribed to a persistent bronchitis.

Shortly before death, a thin, bloody fluid might run from the mouth. In one case the material coughed up about 4 hours before death was unclotted blood with a small admixture of mucus.

Blood in the sputum occasionally came from the nose, but otherwise bloody sputum was always associated with, and sometimes the first evidence of, pneumonia.

From patients with unproductive cough mucoid or mucopurulent material was frequently obtained from the nasopharynx, and examined by the same methods used for sputum.

Direct smears and cultures from 150 cases have been studied. The direct smear of a good specimen of sputum may give a better idea of the bacteriology of the disease than that afforded by culture alone. The smear indicates which organisms predominate, but of course cultures are essential to the complete identification of the organisms seen in the smear, and we made special and repeated efforts to get good specimens of sputum, and from most of the patients several samples were examined. The results are shown in table 1.

TABLE 1
RESULTS OF BACTERIOLOGIC EXAMINATION OF SPUTUM IN 150 CASES OF INFLUENZA

	Cases	Per Cent.
Only saliva obtained	12	8.0
Various organisms, none predominating	35	23.3
Thin, mucoid or mucopurulent material from the nasopharynx.		
Three organisms predominating and in about equal numbers.....	7	4.7
Strept. viridans, B. influenzae, and M. catarrhalis in 3;		
Strept. viridans, B. influenzae, and hemolytic strept. in 3;		
Strept. viridans, B. influenzae, and fusiform bacilli in 1.		
Two organisms predominating or the only ones present	44	29.6
Pneumococcus and B. influenzae in 22,		
The pneumococcus predominating in 13,		
B. influenzae predominating in 9,		
Strept. viridans and B. influenzae in 21,		
B. influenzae predominating in 13,		
Strept. viridans predominating in 8,		
B. influenzae and B. mucosus in 1 case.		
B. influenzae predominating, with a variety of other organisms.....	14	9.3
Pneumococci present in 3 of the 14 cases.		
Hemolytic streptococci predominating	4	2.7
Pure culture of one organism except for a rare colony of M. catarrhalis		
or staphylococcus	34	22.7
Pneumococcus	14 cases	
B. influenzae	12 cases	
M. catarrhalis	3 cases	
Hemolytic streptococci	3 cases	
Staphylococci	2 cases	

Influenza bacilli were found in 87 (63.2%) of 138 cases in which specimens of sputum or nasopharyngeal secretion were obtained. In the direct smear of the sputum, the influenza bacilli were of varied

morphology, appearing in most cases as minute, gram-negative bacilli with rounded ends; usually short; sometimes in pairs like a pneumococcus, but much smaller. In some smears they were so small as to be overlooked easily. In other specimens they appeared as short threads. In sputum which gave a pure culture, they were astonishingly numerous, some being within the leukocytes.

In a bright, reflected light, the colonies on human blood-agar plates were visible as minute, transparent, convex points. By transmitted light, they were invisible. The blood was not changed. Unless the bacilli were numerous in the direct smear, it was very difficult to isolate them. Even when a good growth was obtained on the original plates, the bacilli might not grow in subcultures. Sometimes on the first plate cultures, the influenza bacillus forms long, slender threads like leptothrix, and quite unlike the short, coccoid form. The colonies of this form are slightly larger than those of the short form. It was noted that when the thread form appeared in the direct smears of the sputum, the colonies on the plates contained still longer threads. The same strain may give the two forms in different transfers.

None of the strains grew on hemoglobin-free mediums. In one sputum an organism was found which resembled the influenza bacillus in smears, but in cultures formed larger, opaque colonies and grew on plain agar.

There were frequently found on the plates many colonies of a small streptococcus which did not change blood. From an examination of the plates alone, it would be easy to confuse these colonies with those of influenza bacilli. They were sometimes numerous and occurred early in the epidemic.

When mixed with *B. mucosus*, the influenza bacillus may be overgrown unless transferred early.

Not much difficulty was found in growing the influenza bacillus in pure culture. Other organisms, such as staphylococci, were not necessary to its growth. In fact, the best plates came from sputum in which other bacteria were rare.

Pneumococci of all four types were found in 35 (25.4%) of 138 cases in which sputum or nasopharyngeal secretion was obtained.

PLEURAL EXUDATES

The pus from 8 cases of empyema was examined. In 3 cases hemolytic streptococci, in 4 pneumococci, and in 1 pneumococci and the influenza bacillus were obtained. The blood-tinged, straw colored fluid from another patient contained only influenza bacilli; this exudate was absorbed without drainage.

BLOOD CULTURES

Ninety blood cultures were made in 80 cases. Twenty were taken on the 1st day of the disease; 17 on the 2nd day; 8 on the 3rd day; 7 on the 4th day; 5 on the 5th day; 2 on the 6th day; 3 on the 7th day; 1 on the 9th day, and 1 on the 11th day. The rest were taken on the day of highest temperatures. Aerobic and anaerobic plates and cultures in broth were made in each case and daily transfers were made from the broth to human blood-agar slants.

Seventy-eight of the 90 blood cultures were negative. Blood cultures from 3 soldiers within 24 hours before death gave hemolytic streptococci. Pneumococci were found in the blood in 6 fatal cases, four of them with lobar pneumonia. There were only two positive blood cultures in patients who survived, both of these giving influenza bacilli.

CASES

The mixture of bacteria in the sputum and the negative blood cultures in most cases made the interpretation of the bacteriologic findings difficult. The organism which produces infection does not necessarily predominate in the sputum at the time it is examined. Because mixed cultures are inconclusive, the 34 cases in which practically pure cultures were obtained are of special interest. Included in this group are the cases in which the first plate or two might show a rare colony of *M. catarrhalis* or staphylococcus, but in which the third plate showed many colonies of only one organism. There were cases with only influenza bacilli, pneumococci or hemolytic streptococci not being found; there were pure pneumococcus cases, without influenza bacilli or hemolytic streptococci; and there were cases with only hemolytic streptococci, there being no pneumococci or influenza bacilli. Details are given in regard to a few typical and instructive cases.

CASE 1.—A soldier was admitted October 3 with the diagnosis of influenza. Hemolytic streptococci predominated in the sputum during life, and a blood culture 24 hours before death gave a similar hemolytic streptococcus. Post-mortem examination showed bronchopneumonia and empyema, and hemolytic streptococci and influenza bacilli were cultivated from both lungs and from the trachea. The heart blood gave hemolytic streptococci and *B. mucosus*, the latter most likely being a terminal invader as it did not grow in the blood culture shortly before death.

The nurse who had charge of the soldier was taken sick before he died, and remained on duty until his death when she went to bed with a temperature of 102.6 F. The physical findings were those of bronchitis; the temperature rose to 104.2 that evening, became fluctuating but, on the whole, lower during the next 4 days, being 100.7 on the morning of the 5th day. On the 6th day, it rose to 105; at this time the leukocytes were 5,400. The first specimen of sputum was obtained on this day; it was mucopurulent and tenacious, coming from the lower air passages, and smear showed innumerable small, gram-negative bacilli. The plates showed a pure culture of influenza bacilli, except for a rare colony of staphylococcus. There were no pneumococcus or

streptococcus colonies. On the afternoon of this day the first signs of bronchopneumonia were found in the lower right lobe. The following day, the temperature again reached 105 F., the leukocyte count was 9,700, and the signs of bronchopneumonia in the right lower lobe were more marked. The sputum was mucopurulent and blood streaked. On the 10th day the temperature remained high, and a blood culture was made, but it remained sterile. On the 11th day, the temperature fell to 99, and the patient was much improved. The sputum was still mucopurulent, tenacious, and blood streaked; and it still gave pure culture of influenza bacillus except for a rare colony of staphylococci. On the 17th day the patient complained of a pain in the right side of the chest, the temperature rose to 101.8 and the leukocyte count to 20,000. Coughing was painful and no sputum was procured until the 19th day when a right-sided effusion had relieved the pleural pain. This sputum was greenish, mucopurulent and blood streaked, and instead of pure culture of influenza bacilli, it gave a mixture of influenza bacilli and pneumococci—type III—the pneumococci predominating. On the 21st day, faintly blood-tinged, slightly clouded, straw-colored pleural fluid was aspirated; smears showed some leukocytes and small, gram-negative bacilli, and cultures gave influenza bacilli, no pneumococci being found either in smear or culture. The fluid in the chest was absorbed without drainage.

On the 26th day, when the temperature was normal, the sputum was still mucopurulent with streaks of blood, and cultures gave numerous pneumococci, no influenza bacilli, and a few *M. catarrhalis*, and a streptococcus which did not change blood.

CASE 2.—Patient admitted October 9, in premature labor, temperature of 104 F.; diagnosis, influenza. A living child was born. There were signs of consolidation of the right lung, more marked in the upper lobe, and of a less extensive bronchopneumonia on the left side. The leukocytic count was 6,400; the sputum mucopurulent and tenacious. The predominant organism was the influenza bacillus. There were many colonies of *B. mucosus* and a few of a hemolytic streptococcus. Death occurred on the 5th day after entrance. Blood culture on the last day of life gave pneumococci, type III. Examination 2 hours after death showed the oldest pulmonary lesion to be a confluent bronchopneumonia of about two-thirds of the right upper lobe. Most of this area was gray. Cultures of pneumonic tissue cut out of this lobe after searing the pleural surface gave innumerable colonies of influenza bacilli with only 3 colonies of staphylococci on 3 plates. No other organisms were found in this lobe.

The lower right and left lobes contained discrete dark red patches of fresh bronchopneumonia, cultures of which at the end of 24 hours showed numerous colonies of influenza bacilli, fewer pneumococcus colonies, and scattered colonies of staphylococci, which were overgrown at the end of 48 hours by *B. mucosus*. There was a bilateral thin, turbid, yellow pleural exudate, which contained pneumococci and *B. mucosus*. In the left tonsil was a chronic abscess, the pus containing *B. mucosus* and a hemolytic streptococcus.

CASE 3.—A soldier was admitted October 23, the 5th of illness, with broncho-pneumonia in the lower right lobe, temperature of 103 F. By the 11th day all the lobes were involved in the process. The leukocyte count on admission was 4,200. For the next 4 days the patient was very sick and unable to cough up anything. On the 9th day the temperature was 104, and a specimen of mucopurulent sputum was obtained, which on cultures showed many colonies of influenza bacilli, some colonies of pneumococcus—type II—and a few

colonies of *M. catarrhalis*. The influenza bacilli greatly predominated. This bacteriology of the sputum persisted except for an increase in the relative numbers of pneumococci until the 15th day; after that it was not examined.

Blood cultures on the 7th and 9th days were sterile; on the 11th day the blood gave a growth of pneumococcus, type II. On the 14th day the leukocytes were to 23,300. Although the patient seemed much improved on the 17th day, the blood cultures still contained pneumococci; an ulcerative mitral endocarditis developed, and on the 23rd day a pneumococcus meningitis was diagnosed. Death occurred 4 days later.

Postmortem examination showed bronchopneumonia in the lower and upper right lobes and in the left lower lobe. Cultures of the pneumonic areas yielded influenza bacilli and pneumococci, with occasional colonies of *M. catarrhalis*. Cultures of the vegetations on the heart valves gave only pneumococci.

CASE 4.—A young woman was admitted October 28, with bronchitis and a patch of bronchopneumonia in the lower right lobe, temperature 101.8. F. She said that she had felt badly for a week, but had not been in bed; leukocyte count 2,700; no spontaneous cough or expectoration; a forced cough resulted in a small amount of clear mucus which contained in order of frequency, hemolytic streptococci, influenza bacilli, and *M. catarrhalis*. Two days later the sputum gave the same result. Five days later, the temperature being normal, hemolytic streptococci predominated in the sputum, and no influenza bacilli were found. Blood culture on the day of admission was negative; on the following day, when the temperature had risen to 104, blood culture showed influenza bacilli. There were no complications, the temperature falling to normal 2 days later.

CASE 5.—A nurse became ill October 18, with lobar pneumonia on one side and of bronchopneumonia on the other side; leukocyte count was 26,300; sputum scant, slightly yellow, tenacious, the cultures showing numerous colonies of influenza bacilli (leptothrix form), and many but less numerous colonies of pneumococcus—type III. Blood culture on the 1st day of the disease gave the leptothrix form of the influenza bacillus; no pneumococci. This patient recovered quickly and completely.

CASE 6.—A soldier became ill on a train and entered the hospital October 26, with a temperature of 101 F.; leukocyte count, 4,800; slight cough but very little sputum; highest temperature was 101.8. He did not develop pneumonia and recovered promptly. A small amount of clear, tenacious sputum containing some coal pigment was obtained the day of admission, smears of which showed some leukocytes and many small gram-negative bacilli. The cultures yielded innumerable colonies of influenza bacilli and a few colonies of staphylococci. There were no colonies of pneumococci or streptococci. Two days later the temperature being normal, a small amount of clear mucus expelled by forced cough still contained innumerable influenza bacilli, and also some streptococci of both viridans hemolytic types.

CASE 7.—A woman was admitted October 10, with diagnosis of influenza and bronchopneumonia; temperature, 104.2 F.; leukocyte count, 6,600. For 2 days the patient was quite sick; then she began to improve and at the end of 12 days the temperature was below 100. It went as low as 98.2, but frequently rose to 99 and a fraction until the time of discharge 10 days later. This incomplete recovery was associated with bronchitis. There was frequent cough, and abundant mucopurulent sputum. During all this time the sputum was

practically a pure culture of influenza bacilli except for an occasional colony of staphylococci and a few hemolytic streptococci in the last specimen examined before the patient went home.

CASE 8.—A nurse had a light attack of illness during October when the epidemic was at its height, but was not particularly sick and worked while she had a persistent dry cough and a pain in the right chest. On November 6, she went to bed though her symptoms were not much worse than they had been for some time. Temperature, 101.6 F.; leukocyte count, 14,400; a slight friction rub over the lower right lobe; cough troublesome, and only after considerable effort was any sputum obtained, which seemed to come from far down in the chest. Cultures of the mucopurulent sputum yielded numerous colonies of influenza bacilli, many of *Strep. viridans*, a few of *M. catarrhalis*, and an occasional colony of hemolytic streptococci. Another specimen obtained after the temperature had been normal 15 days, gave the same result with the addition of a few staphylococci. The patient continued to cough with diminishing expectoration for 3 weeks after the temperature became normal. The throat and nose cultures were negative for influenza bacillus while the sputum was positive. At the time this nurse sought treatment for the persistent cough and pain in the side, there was a limited epidemic among the nurses, and the patient volunteered the information that all of the nurses who were sick at that time had been associated with her.

CASE 9.—A man was brought into the hospital very sick, November 3. He had been ill at home a week. The lower left lobe was solid; he was delirious and died within 48 hours. Blood culture 20 hours before death contained pneumococcus—type III. The sputum on admission was mucopurulent and tenacious; it contained no influenza bacilli and no streptococci, the predominant organism being pneumococci—type III. There were some colonies of a small streptococcus which did not change blood, and a few colonies of staphylococci. On postmortem examination there was found an extensive confluent bronchopneumonia in all the lobes, most marked in the lower, and the pneumonic areas gave no influenza bacilli, only the pneumococcus in practically pure culture.

CASE 10.—A colored woman was admitted December 10; temperature of 100.8 F.; grunting expiration; left lower lobe solid; a leukocyte count of 20,400; the sputum mucopurulent, rusty and tenacious, gave a pure culture of pneumococcus—type I; a blood culture on admission gave the pneumococcus. The patient died 2 days later.

COMMENTS ON CASES

The first of the cases is of interest because the nurse presumably contracted the disease from the soldier in whom hemolytic streptococci predominated and in whom pneumococci were not found. Hemolytic streptococci which predominated in the sputum and were present in the blood culture of the soldier were found in neither sputum nor blood of the nurse; while pneumococci, which appeared in the nurse's sputum, was not found in the soldier. The only organism found in both was the influenza bacillus. The second point is that the influenza bacillus, practically pure in the sputum, preceded the

appearance of the pneumococcus, and that once the pneumococcus appeared, it was not long before it had entirely supplanted the influenza bacillus. The rise in leukocytes from 5,400 to 20,000 might be attributed either to the invasion of the pneumococcus or to the involvement of the pleura by the influenza bacillus.

The second case had a complicated bacteriology including the influenza bacillus, the pneumococcus, and *B. mucosus*. The presence in pure culture of the influenza bacillus in the oldest bronchopneumonic area indicates that its invasion of the lung antedated that of *B. mucosus* and pneumococcus. It is probable that the hemolytic streptococcus and *B. mucosus* found in the sputum were present in the chronic tonsillar abscess before the onset of influenza.

In Case 3, the pneumococcus and the influenza bacillus were present in the sputum on the 5th day of the disease. There is nothing to indicate which, if either, was the primary invader. The pneumococcus did not appear in the blood until the 11th day of the disease.

Case 4 was a typical, fairly mild case of influenza with leukopenia and a small patch of bronchopneumonia. This patient is one of the two who had positive blood cultures and recovered. It is of interest because the influenza bacillus was found in the blood culture when it was not the predominant organism in the sputum.

Case 5 is noteworthy because the bacteriology of the sputum and the physical findings indicated a combination of bronchopneumonia and lobar pneumonia; it is the other of the two cases in which recovery occurred after influenza bacilli had been demonstrated in the blood.

Case 6 was a mild case of influenza without pneumonia, with leukopenia and many influenza bacilli, but no pneumococci or streptococci in the first specimen of sputum.

Case 7 was characterized by bronchitis with mucopurulent sputum following influenza. The patient was expelling large numbers of influenza bacilli at the time of her discharge, and might continue to do so for a long time.

Case 8 may be classed as a carrier of influenza bacilli since the sputum contained the bacilli at least 15 days after the temperature was normal.

Case 9 is one of fatal bronchopneumonia in which only pneumococcus—type III—was demonstrated. The short time required for this organism to overgrow the influenza bacillus in the sputum of Case 1 suggests that pneumococcus III may not be the primary invader, even in those cases in which it is found in practically pure culture.

Case 10 is an example of what has been diagnosed in this series as typical lobar pneumonia. The patient had grunting expiration, a lobar consolidation, a high leukocyte count, and pneumococci in pure culture in the sputum and blood. The two specimens of sputum in which staphylococci appeared in pure culture and the three which yielded a pure growth of catarrhalis were examined late in the disease. These organisms were not found in pure culture in the sputum early in the disease, and were not found in the blood cultures. They doubtless were secondary invaders.

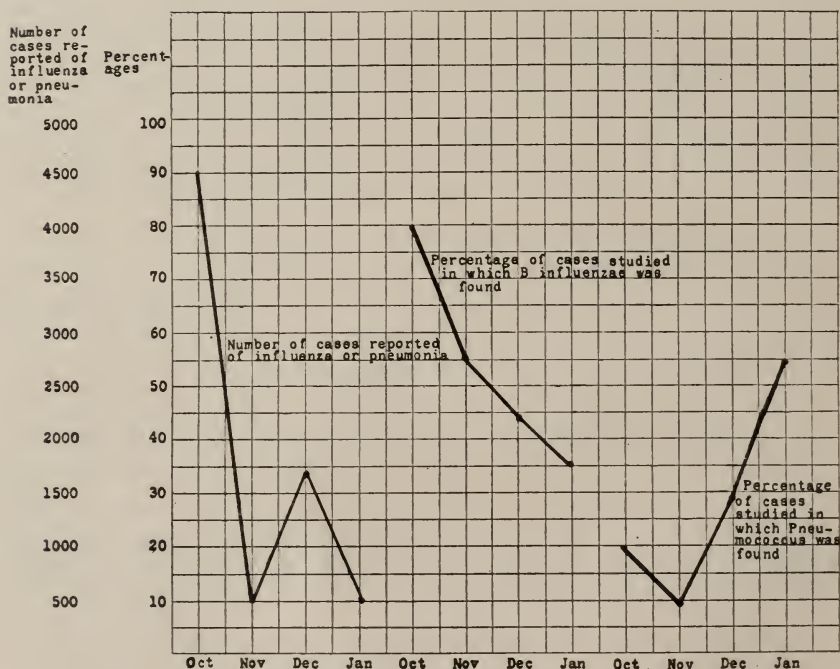


Chart 1.—Relation of results of bacteriologic studies to influenza and pneumonia in Chicago.

DISCUSSION

Green-forming streptococci and streptococci which did not change blood were found frequently in the sputum, but never in pure culture, and were not isolated from the blood or from pneumonic areas. Just what part they play in the course of the infections is undetermined.

Hemolytic streptococci were few or absent in all but 11 cases. The four patients in whom they predominated in the sputum, and the three

who gave streptococci in pure culture died. Empyema was present in three of the fatal cases. The specimens of sputum which gave pure cultures of hemolytic streptococci were obtained within 48 hours before death.

The late appearance of hemolytic streptococci in the sputum and in the blood cultures, with the relatively infrequent occurrence in the series indicates that, however important they may be in determining the course of the disease, they were not the primary invaders.

The organisms most frequently found were the influenza bacillus and pneumococcus. They were found together in 47 cases. The influenza bacillus was supplanted by the pneumococcus in two cases and appeared before the pneumococcus in another case. In no instance did the influenza bacillus appear after the pneumococcus.

The influenza bacillus was found in 79% of the cases during the 1st month of the epidemic, and the highest number of cases giving a pure culture of influenza bacilli was found during the same month, while the number of cases showing pure cultures of pneumococci increased from 2 in October to 12 in January.

TABLE 2
RELATIONS OF RESULTS OF BACTERIOLOGIC EXAMINATION TO INFLUENZA AND
PNEUMONIA IN CHICAGO

	October	November	December	January
Number of cases of influenza and pneumonia reported in Chicago	43,375	5,991	17,691	6,232
Percentage of cases studied in St. Luke's Hospital in which <i>B. influenzae</i> was found.....	78.9	54.5	43.3	36.3
Number of cases in which <i>B. Influenzae</i> was found in pure culture	7	0	4	2
Percentage of cases studied in St. Luke's Hospital in which pneumococcus was found.....	19	9	28.3	54.4
Number of cases in which pneumococcus was found in pure culture	2	1	8	12
Number of cases in which hemolytic streptococci were found in pure culture	0	0	3	0

Table 2 shows the relation of the epidemic in Chicago to the occurrence of pure cultures of pneumococci, influenza bacilli, and the hemolytic streptococci. The incidence of the influenza bacillus follows that of the epidemic most closely. The highest point for each is in October. The pneumococcus shows a rise during the recrudescence of the epidemic in December, and continues to rise in January, though the epidemic curve fell in that month. The figures include cases studied till the end of January (cases observed later than January 15, are not included in the 150 cases of table 1). The figures for the epidemic are the number of cases of influenza and pneumonia reported to the Chicago Health Department.

Table 2 also shows the relation of the percentage of cases in which influenza bacilli and pneumococci were found in either pure or mixed culture to the incidence of the epidemic. It is significant that the figures showing the incidence of the influenza bacillus were highest in October and fell steadily while the pneumococcus figures ran relatively low in October and rose as the frequency of the influenza bacillus fell. In general, the incidence of the influenza bacillus corresponds to that of the epidemic.

The disease clinically recognized as influenza was characterized by a leukopenia in the uncomplicated cases in contrast to the leukocytosis of lobar pneumonia. The average leukocyte count of both complicated and uncomplicated cases during the month of October when the epidemic was at its height was 6,400. The average leukocyte count when the influenza bacillus occurred in pure culture was 5,400. The average leukocyte count when pneumococcus was found in pure culture was 15,200.

As noted, types I, II, III and IV of pneumococci were identified. During October the influenza bacillus was found in 45 cases, and pneumococci in 11; eight of the pneumococcus strains isolated early in the epidemic belonged to type III, and the pure cultures of pneumococcus obtained in the month of October in two cases were both of this type. Later there were more cases of the other pneumococcus types and rarely one of type III. In the routine blood cultures in pneumonia during the winter of 1917-1918, the influenza bacillus (leptothrix form) was encountered once in pure culture in the blood from a case of bronchopneumonia with pleurisy. In April, 1918, the influenza bacillus was grown in pure culture from the pleural exudate of a soldier who had had bronchopneumonia.

Cole and MacCallum¹ in the pneumonia epidemic at Fort Sam Houston, and MacCallum² at Camp Dodge, found the influenza bacillus frequently in the late winter and early spring of 1918. Dick³ found it to be the predominant organism in the sputum of 6% of the cases of pneumonia studied in the base hospital at Camp Pike in February and March, 1918. Tunnicliff⁴ reports influenza bacilli in 58% of the sputum cultures at Camp Meade in the early fall of 1918. Though Cole and MacCallum found the influenza bacillus frequently, the bac-

¹ Jour. Am. Med. Assn., 1918, 70, p. 1146.

² Ibid., 1918, 71, p. 704.

³ Ibid., 1918, 70, p. 1529.

⁴ Ibid., 1918, 71, p. 1733.

teriologic picture of the epidemics they studied was dominated by hemolytic streptococci. The epidemic at Camp Pike described by Dick was characterized by the predominance of both hemolytic streptococci and a streptococcus which had no effect on blood. The epidemic studied by Tunncliffe differed from the others by the predominance of a green-producing streptococcus.

Whatever the etiology of influenza may be, secondary infection plays a most important rôle. It seems that in any one group of persons closely associated, the secondary invaders may be quite constant, but different in various groups; so that the results of the bacteriologic studies of different groups are conflicting. It is even possible that an organism entering the epidemic as a secondary invader may acquire such an increase in virulence that it is able to continue epidemic infection after the original cause has disappeared.

SUMMARY

In this series of cases drawn chiefly from the civilian population and belonging to no one group of people, *M. catarrhalis*, staphylococci, hemolytic streptococci, various types of pneumococci, including type III,⁵ and *B. influenzae* were all found; usually in mixed culture, but sometimes singly. Of these organisms the influenza bacillus was the one most constantly present in October, when the epidemic was at its height, and the only one found in blood cultures of patients who recovered.

The leukocyte count in the cases of pure influenza bacillus infection was low, corresponding to the leukopenia regarded as characteristic of epidemic influenza.

The influenza bacillus may be present in pure culture early in the disease and be supplanted by the pneumococcus later. In this series none of the patients with a pure influenza bacillus infection died.

⁵ This organism appeared as diplococci in direct smears of the sputum, and formed large, flat, moist colonies on blood-agar plates. The colonies tended to coalesce, resulting in ameboid shapes. The organism was soluble in bile, but dissolved more slowly than the other types. It fermented inulin, and was agglutinated by type 3 pneumococcus serum.

CLINICAL OBSERVATIONS ON INFLUENZA WITH SPECIAL REFERENCE TO THE BLOOD AND BLOOD PRESSURE *

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This is a report of observations on influenza at the Michael Reese Hospital of Chicago during the epidemic of 1918. There were 546 cases of influenza admitted between September 20 and December 31, 185 males and 361 females. The ages varied from 3 months to 80 years, the greatest age incidence being between 20 and 30 years (chart 1). In 55 cases an exhaustive daily study was made of blood, urine, and blood pressure. In all the other cases the symptoms, physical findings, course, and results of occasional examinations of the blood, urine, etc., were recorded.

ONSET

Good histories of many patients were obtained, especially of nurses taken ill while on duty. Except for minor variations, the onset of the disease was much the same, the attack usually starting suddenly with headache in the frontal region; the others described it as diffuse. Another constant symptom at the onset was pain or ache in the lumbar region, more marked in the female patients. In addition there were vague pains in the limbs, and often in the chest. Fever was constant, usually ranging between 103 and 104 F., although there were a few cases in which the temperature ran as high as 106 F. at the beginning. Thirty-five of 230 patients gave a history of a chill at the onset; in some there was only a "shivery sensation." A few patients complained of sore throat as one of the first manifestations. Several spoke of pains in the right lower quadrant of the abdomen. In a number of women menstruation came on too early, occurring on the first day of the disease.

The symptoms mentioned usually lasted from several hours to a day or so, when a cough would appear, dry and painful at first. A few

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* Influenza Investigations U. S. Public Health Service.

patients also complained of nausea at this time. In the majority no nasal symptoms were present, although sneezing was noted in several; in a few there was a slight discharge from the nose, and epistaxis occurred in a small number. In almost all, the edges and tip of the tongue were markedly red, the rest heavily coated. Most of the patients complained of soreness of the trachea, pointing out that the pain was not in the throat but lower down.

COURSE

From the third day the cases may be divided into two groups—mild and severe. As a rule the mild cases were uncomplicated, the severe complicated usually by bronchopneumonia, less often by

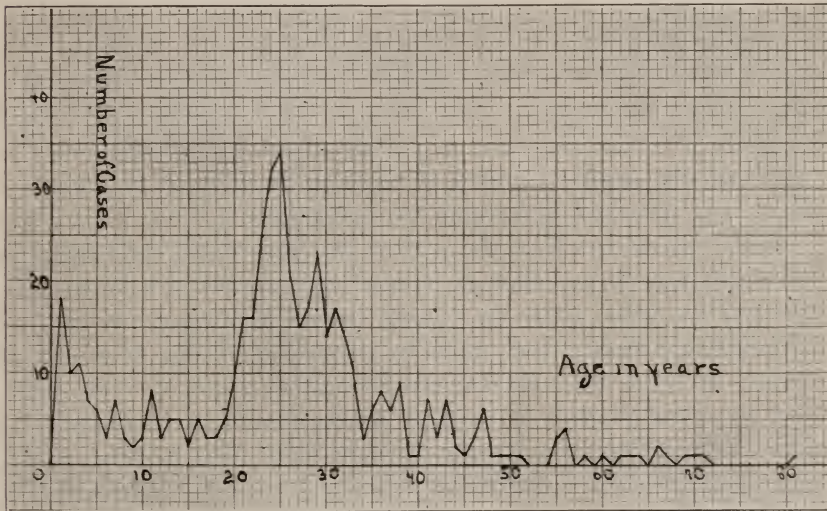


Chart 1.—Distribution according to ages of 546 influenza patients.

empyema or otitis media. In the mild cases the symptoms subsided gradually, the headache being less severe on the 3rd day, the temperature reaching normal on the 5th or 6th day, if not earlier. Many patients, however, complained of dizziness and general weakness for several days and even weeks afterward.

In the severe cases the temperature on the 3rd day would be 102 or 103 F.; or if it had dropped on the 3rd day, which happened in a few cases, it would rise again; the cough, at first dry, would become

productive and painful, and this was usually the signal of a broncho-pneumonia, as râles would develop and the general condition would become worse. The cough usually continued until the end of the attack. There were, however, cases in which the cough subsided early. In cases that recovered, the fever usually ended by lysis; in the fatal cases the temperature remained high, sometimes it was 106 or 107, in one case even 109 F. Definite cyanosis was present in eight cases of pneumonia that recovered; in all the fatal cases cyanosis was marked and accompanied with unconsciousness and muscular twitchings. Death usually occurred on the 6th to 11th days of the disease, in a few cases on the 3rd or 4th day.

PHYSICAL FINDINGS

In uncomplicated cases there were few physical changes. The tongue was coated, the pharynx slightly congested, and occasionally conjunctivitis was present. The heart, as a rule, showed no change, the pulse, however, was slow in proportion to the temperature. An occasional râle would be heard at times at the base of the lungs; in many cases, however, râles could not be detected.

In the pneumonic form the patient was prostrated, the respiration rapid, although slower than in ordinary pneumonia; the temperature high and the pulse slow; the tongue furred, the edges and tip red; the cough distressing, and often in paroxysms ending in a sort of whoop. In some cases dulness was difficult to detect over the lungs; when present it was not as fixed as in other forms of pneumonia, but often changed from day to day, or even from hour to hour; nearly every case revealed crepitant or subcrepitant râles in some part of the chest; bronchial breathing was less frequent and often heard over small patches only of lung. The most frequent localization of pneumonia was both bases, next the left lower lobe, then the right middle lobe or prevertebral region, the right lower lobe, and least frequently the left and right upper lobes. In the last stages of the disease, however, especially in the fatal cases, moist râles would be heard all over the chest, giving the impression that no spot in the lung was free.

Rigidity of the right rectus abdominalis muscle was found in some patients and not in others. Meningeal symptoms were absent, unless headache and unconsciousness were considered as such.

TABLE 1 (Continued)
BLOOD COUNTS IN INFLUENZA

Cases	Day of At- tack	Hemo- globin	Red Cor- puscles	Leukocytes							Remarks	
				Total Num- ber	Neu- tro- phils	Small Mono- nu- clears	Large Mono- nu- clears	Trans- ition- als	Eo- sino- phils	Baso- phils		
11	1	85	5,200								Pneumonia developed
	2	85	5,200,000	3,200	84	16						
	3	85	4,640,000	6,000	50	48	2					
	7	75	4,200,000	10,200	80	18	2		
	8	85	5,200,000	16,200	98	2						
12	1	3,400								Otitis media developed
	2	..	3,120,000	5,400	54	24	8	2				
	3	90	3,640,000	3,400	62	22	14	2				
	5	85	3,880,000	3,200	48	52						
	7	10,600	78	18	4		
13	1	4,000								Pneumonia
	2	95	5,080,000	3,400	52	44	4					
	3	95	3,600	58	36	6					
	4	95	4,040,000	3,200	54	44	2		
	5	80	3,680,000	6,200	46	46	8					
	6	80	4,200,000	4,200	60	34	6					
	8	80	4,720,000	7,000	88	12						
14	1	4,900	81	18	1					Pneumonia
	2	4,400	66	24						
	3	85	4,800	64	30	4	2				
	4	85	5,600,000	6,000	54	45	1		
	5	85	6,000	64	32	4					
	6	95	5,160,000	5,800	48	48	4					
	10	95	4,400,000	14,000	92	4	4					
15	1	85	5,800	82	18						Pneumonia
	2	90	4,400	66	34						
	3	85	4,120,000	5,800	70	24	4	2				
	5	85	4,320,000	2,800	70	22	8					
	10	80	4,400,000	9,000	76	22	2		
	12	85	6,200,000	18,000	82	16	2					
16	1	85	3,040,000	5,000	22	74	4					Child
	2	85	4,000,000	7,400	36	64						
	4	85	4,040,000	4,800	28	72						
	6	80	4,120,000	8,200	30	64	6					
	8	80	6,400	64	24	10	2				
17	1	95	4,000,000	6,800	27	66	7					Child
	2	95	7,200	48	50	2					
	4	85	5,680,000	7,400	36	62	2					
	6	85	5,160,000	3,600	28	64	6	2				
	8	85	5,200,000	15,200	37	63						
18	1	85	5,600,000	6,900	42	56	2					Child
	2	85	6,160,000	7,200	60	36	4					
	4	85	4,200,000	7,200	56	30	10	2	2			
	10	85	6,300,000	16,100	56	28	9	6	1			
19	1	90	6,100,000	9,000	50	34	10	6				Child
	2	85	4,720,000	4,600	16	80	4					
	4	85	5,640,000	8,400	46	50	4					
	10	95	6,550,000	8,700	40	40	10	8	2			

* The 19 cases of this table are illustrative of the blood findings in the 55 cases studied.

BLOOD (TABLE I)

Blood counts and blood pressure determination were made about 11 a. m.. Wherever possible, blood was examined some time after

the patient had recovered, for comparison. For the differential counts carbolthionin and Wright stains were used.

Nearly all the uncomplicated cases showed leukopenia and lymphocytosis. At the onset the leukocytes numbered from 4,000 to 6,400 per c.mm. After the 1st day they began to decrease in number, being 2,000 to 3,000 on the 2nd, 3rd and 4th days. The leukopenia persisted until the patient was well on the road to recovery when the count would rise to 9,800-14,000. This leukocytosis usually developed between the 6th and 8th days of the attack.

The differential count was also characteristic. There was a change from the normal differential count (sometimes there was a neutrophilia on the 1st day) to a relative lymphocytosis on the 2nd or 3rd day. The rise in lymphocytes which was gradual reached at times 50-55%. This condition persisted until the patient recovered, when the neutrophils rose to 67 or 70%. This, however, was only temporary, the count on the next day or two often showing again a relative lymphocytosis.

The development of complications sometimes was associated with a change from leukopenia with relative lymphocytosis to leukocytosis with relative neutrophilia (Table 1, Cases 8-12). In other cases, however, complications did not change the blood picture; this was true especially in some cases that developed pneumonia (Table 1, Cases 13-15). Even in those cases in which complication was followed by a leukocytosis the count was not as high as in many pyogenic infections. In fact, some of the cases of pneumonia gave a higher leukocyte count after the pneumonia subsided than during the active stage. In three fatal cases leukocytosis (12,000-16,000) with neutrophilia was found before death, with 86-98% neutrophils.

No basophils, and only a very few eosinophils were found. As a rule, there were no transitional cells in the blood. Occasionally, however, the smears would contain many transitional cells.

In the uncomplicated cases the red cells were not affected, either in number or structure. In cases with pneumonia a low red count might be obtained toward the end. Nucleated red cells were not found. The hemoglobin was not affected in any of the cases.

BLOOD PRESSURE

The blood pressure was found to give a fairly uniform curve. Normal at the onset, the pressure, both systolic and diastolic, but especially the latter would gradually fall. For instance, a patient with

a systolic pressure on the 1st day of 130 and a diastolic pressure of 90 would show the following variations from day to day; 2nd day, systolic pressure 120, diastolic 70; 3rd day, systolic 110, diastolic 55; 4th and 5th days, systolic 105 and diastolic 50, the pressure now usually remaining constant for several days after which it would begin to rise again. In one case the diastolic pressure fell as low as 35. The fall in pressure was observed in uncomplicated as well as in complicated cases.

URINE

In uncomplicated cases the urine, as a rule, was normal, but occasionally a trace of albumin was noted. In most cases that developed pneumonia the urine showed more or less albumin, and in some there were hyaline and granular casts. In the fatal cases there were practically always many granular and hyaline casts 2 to 3 days before death. In two cases hematuria developed 2 days before death. Acetone was absent in most cases.

THE SPUTUM

The sputum was tenacious at the beginning of pneumonia and usually remained so for 3 or 4 days, after which it became frothy. In some cases, however, it remained tenacious for a much longer period. The color of the sputum was yellow at first; later, however, it became rusty and often looked as though it consisted entirely of blood, especially in the fatal cases. Microscopically, the sputum usually showed pneumococci, streptococci and staphylococci. Influenza-like bacilli were seen in only a few cases.

COMPLICATIONS

Besides pneumonia, the cases presented comparatively few complications. There were 12 cases of otitis media in the 546 cases of influenza observed, 8 of empyema (hemolytic streptococci in 50%), 1 of lung abscess, 1 of hemoptysis, 3 of myocarditis, 2 of hematuria, 2 of retention of urine, 3 of jaundice, 1 of profuse uterine hemorrhage, 19 of premature menstruation, 1 of cervical cellulitis, 1 of multiple abscess, 2 of maculo-papular rash, 3 with decided muscular twitchings and 2 of delirium. Psychosis developed in 2 cases.

PROGNOSIS

Of the 454 adults treated in the hospital, 80 died, approximately 20%, and of the 92 children, 10 died, approximately 11%. The

greatest number died in October, the early part of the epidemic. Of the 80 fatal adult cases, 43 were women and 37 men. As eight of the fatal cases in women were complicated by pregnancy, and as the rate of admission of women compared to men was 2 to 1, it would seem that more men died from influenza than women. The highest mortality in both cases was between the ages of 20 and 30.

In practically all the fatal cases pneumonia was present. Rest in bed from the beginning of the attack seemed to have a favorable effect. Most of the nurses, who went to bed immediately after taking sick, recovered; while of those who remained on duty for 2 or 3 days many died. The fever seemed to have no direct relation to the outcome, some patients with a temperature of 106 F. at the beginning recovered, while others who ran relatively low temperatures died.

Of the 15 pregnant women, 8 died, 5 being in the 7th month and 3 at full term. Five of the 8 that died miscarried before death. Of the 7 that recovered, 3 were at full term, 2 in the 8th month, and 2 in the 7th. Existing heart lesions increased the danger from influenza. Cyanosis is an unfavorable prognostic sign and was present in practically all the fatal cases.

INFLUENZA IN CHILDREN

In 92 cases observed, the symptoms of influenza in children differed from those of adults in degree rather than kind. Vomiting was more frequent in children. The temperature was lower than in the adults, particularly in the fatal cases. Sneezing at the onset was more frequent, also coryza and conjunctivitis. Epistaxis was common on the 2nd or 3rd day. In practically every case the throat was congested, and sometimes even fiery red. The cough was more spasmodic than in adults, resembling whooping cough closely. Expectoration was noted in one case only. Cyanosis was observed only in the fatal cases.

The course of the disease in children, like that in adults, was mild or severe, that is, there were two groups of cases, those that did not develop pneumonia and those that did. The physical findings in the former were practically nil, except for a pharyngitis which was aggravated by irritation from the cough. The rash noted in a few cases could be traced to belladonna or serum; it usually occurred late in the attack or after it had subsided. In twelve cases I noticed, opposite the second molar, a group of shining round vesicles closely aggregated, the mucous membrane all around being markedly congested. This condition lasted from 3-4 days.

In the severe or pneumonic form the physical findings varied, the only constant one being subcrepitant râles over the affected area. Bronchial breathing and localized dulness were made out in about half the cases, the dulness frequently traveling from one spot to another, the signs varying at different times of the day. Pneumonia was most frequently localized at the base of the lungs and next at the right middle lobe. Herpes labialis occurred more often in children than in adults.

Allowing for the normal differences in the blood of children and of adults, the changes in the blood in influenza seem to be about the same, namely, leukopenia and lymphocytosis, the leukocyte count running from 6,000-8,400, which of course is a leukopenia for children (Table 1, Cases 16-19). A leukocytosis takes place on recovery. In most uncomplicated cases the differential count showed a distinct lymphocytosis, although in a few it was normal. The red blood cells were usually below five million.

The urine in children as a rule was normal, albumin generally absent.

There were three cases of otitis. Meningitis was not observed, and symptoms of meningeal irritation were infrequent. The result in children was more favorable than in adults — of 92 children, 10 died, approximately 11%.

SUMMARY

The greatest number of the cases in this series occurred between the ages of 20 and 30, corresponding to the experience of most observers. In addition there were, current opinion to the contrary notwithstanding, a good many cases in very young children. Headache, pain in the lumbar region, and fever, were constant symptoms at the onset. Most patients also complained of soreness of the trachea. Only a few patients complained of sore throat. Chills, described by some as of frequent occurrence, were noted only occasionally.

In adults, I observed only two maculopapular rashes, both of which resembled drug rashes and occurred after the administration of belladonna and bromids. Meningeal symptoms, reported by some, were absent. The blood, described usually as showing a neutrophilia, in the cases I studied gave a lymphocytosis at certain stages of the disease, with leukocytosis and neutrophilia on recovery. The blood pressure gave a regular downward curve. In 12 children I observed an endanthem in the mouth which may be of significance.

The cases studied do not justify a classification into respiratory, gastro-intestinal and nervous types, as all cases were essentially respiratory. There were fewer nervous manifestations in the influenza with bronchopneumonia than in ordinary pneumonia.

The diagnosis of the disease, I believe, may be made from the sudden onset, headache, backache, pain in the limbs, fever, leukopenia, lymphocytosis, slow pulse and low blood pressure. These symptoms should be sufficient to differentiate influenza from the ordinary respiratory infections with conjunctivitis, sore throat, coryza, and leukocytosis.

From a study of the blood the suggestion arises that two types of pneumonia may complicate influenza; one associated with leukopenia, and hence possibly caused by the same organism that produces the original influenza, and the other with a leukocytosis and due in all probability to secondary infection by better known organisms.

OBSERVATIONS ON THE BACTERIOLOGY OF INFLUENZA

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These observations were made on (A) patients affected in an epidemic in the Student Army Training Corps of the University of Chicago which I have described elsewhere;[†] (B) civilian influenza patients in various hospitals in the vicinity of the University of Chicago; (C) patients, mainly University students, who developed influenza in December, 1918-February, 1919, after the main Chicago epidemic had subsided, and (D) cases of tonsillitis, "colds," and other respiratory tract affections occurring during and subsequent to the epidemic. An attempt was made in Groups A, C and D to study a few cases in great detail, making frequent and very thorough bacteriologic examinations. In one case of influenza pneumonia, for example, observations extended over a period of 36 days until convalescence was complete and the patient discharged, and included sets of plate cultures on 25 different days.

One object especially in view was the determination of the relative frequency and abundance of the Pfeiffer bacillus in the upper respiratory tract of persons suffering from influenza and from common, nonspecific respiratory tract infections. Another was a series of similar observations on the diplo-streptococcus described by Mathers.¹ Variations in the nose and throat flora throughout the illness of one and the same individual were also particularly noted.

Methods.—In the majority of cases swabs have been made from the nose, tonsils and nasopharynx; the nasopharynx swabs have been obtained by the Mathers' bent wire method as used in meningococcus-carrier work.² Nasal swabs usually failed to give results materially different from the others and in the later cases were omitted.

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* Influenza Investigations, U. S. Public Health Service. This paper also appears in the Public Health Report for July, 1919.

† Proc. Inst. of Med. of Chicago, 1919, 2, 135.

¹ Jour. Amer. Med. Assn., 1918, 71, p. 1733.

² "Standard Technique of Meningococcus Carrier Detection," published by the Medical Depts. of the Army and Navy and the U. S. Public Health Service.

The swab has been smeared as soon as made on freshly poured warm blood-agar plates, which are kept warm en route to the incubator. The meat infusion agar has been prepared with 2% peptone and made neutral to phenolphthalein; it has proved well adapted to the growth of pure cultures of the Pfeiffer bacillus. Human blood and sheep blood (5%) have been chiefly used; no material difference was noted. From the smear radiating streaks are made with a platinum loop² and crossed after burning off the needle. Well separated colonies are usually obtained in this way. It has been found advantageous to have plates poured on the day they are used or at most not more than 24 hours before use. Special care is taken to keep the air of the incubator moist. If the surface of the plate is dry when inoculated, or if the air of the incubator is not sufficiently moist, growth of the Pfeiffer bacillus may be unfavorably affected. The plates have been examined after 24 and again after 48 hours, always using a hand lens. In the present series particular attention has been paid to the occurrence of the Pfeiffer bacillus and of the green-producing streptococcus isolated by Mathers.¹ Other bacteria when present in noteworthy numbers have also been isolated and examined. As a rule several colonies from each plate have been circled and fished with a platinum loop to a fresh plate; if this second plate is a pure culture, appropriate diagnostic tests are made. Heated blood-agar plates on which the Pfeiffer bacillus grows with special luxuriance have been used in many cases together with the standard blood-agar plates, but their use has not been essential for the isolation of the Pfeiffer bacillus which has grown freely on the plain blood-agar medium we have used. The well known favoring influence of hemolytic colonies on the growth of the Pfeiffer bacillus has been often observed; it has also been noted that the colonies of certain nonhemolytic bacteria have a similar stimulating effect which is quite as marked. In cases in which the growth of the Pfeiffer bacillus on the first plates was scanty after 24 hours we have sometimes obtained much larger colonies by streaking the plates with one of the growth-favoring organisms and incubating for 24 hours longer. Occasionally the original plate has been too crowded for proper examination and if so, a second plate has been made from it before fishing. Two points have seemed of special importance in the successful isolation of the Pfeiffer bacillus, particularly when this organism is present in small numbers: (1) the incubation of the plates for two or three days, and (2) the use of the hand lens.

Sputum has been examined when it was possible to obtain it. Direct stains have been made and also smears on blood-agar plates in the usual way. When, as is often the case in uncomplicated influenza, sputum in washable quantity is unobtainable, we have had the patient cough directly on a blood-agar plate. Little flakes of mucus are sometimes discharged and after 18-24 hours the growth surrounding these mucous droplets is transferred with a loop to a fresh plate. In this way the Pfeiffer bacillus has been isolated several times when the other sources of examination failed to yield it.

Blood Cultures.—Blood drawn from the elbow vein (about 10 cc in each case) was added to warm broth (200-500 cc) and incubated for about a week. The observations comprised eleven of uncomplicated influenza and eight of influenza pneumonia. In the plain influenza cases blood was drawn on the 1st day in two, on the 2nd day in five, and in one each on the 3rd, 4th, 5th and 7th days; in the pneumonia following influenza the blood cultures were made, respectively, on the 4th (2), 5th (1), 6th (1), 7th (2), 8th (1) and 12th (1) days. All were sterile.

The Pfeiffer Bacillus.—The small translucent colonies on blood agar can often be identified with a high degree of certainty with the hand lens, particularly if they are numerous, in which case the heaping-up around large colonies of staphylococci and other bacteria is highly characteristic. For definite identification it has been our practice to transfer single isolated colonies to a fresh plate; if typical growth was obtained, failure to grow on plain agar together with characteristic morphology and gram-stain reaction were regarded as sufficient identification marks. A luxuriant slimy growth can be obtained on agar prepared with heated blood, but except for obtaining large quantities of material for inoculation experiments this medium presents no noteworthy advantages over the ordinary clear medium. It is not particularly well adapted for isolation.

It is of interest that a medium made without meat infusion, meat extract or peptone—simple agar dissolved in physiologic salt solution—to which 5% of blood is added in the usual way, yields a scanty but undoubted growth of the Pfeiffer bacillus.

For preserving cultures it is safest to make daily transfers, though if the air of the incubator is kept moist, the Pfeiffer bacillus can retain its vitality for a considerable period in the incubator. We have frequently obtained growth from tube cultures that had been in the incubator for from two to three weeks. The heated blood medium is better than the ordinary blood medium for maintaining vitality.

The Mathers Coccus.—This organism was isolated by the late Captain Mathers during the influenza epidemic in September, 1918, at Camp Meade. A culture kindly furnished me by Dr. Tunnicliff possessed the characters described in her paper.³ It resembles the ordinary mouth streptococcus in some of its characters, but the colonies on blood agar are much like those of the pneumococcus, although as a rule larger, moister and more confluent. It is gram-positive, usually with pointed ends and in pairs. It is not soluble in bile, and most strains ferment neither inulin nor mannite. Morphologically and in colony growth it is closer to the pneumococcus than to the streptococcus, but the fermentation characters are those of the ordinary mouth streptococci.

A coccus with these characters was found in a large percentage of the cases examined, not infrequently in practically pure culture especially in cultures from nasopharyngeal swabs. One hundred and eight strains obtained at different times from 44 different cases were subjected to careful examination. All were gram-positive, had the morphology described above and gave a heavy, moist, green, confluent growth on blood agar. Table 1 shows their close relationship to strains of *Streptococcus buccalis* (Blake's classification) isolated in this same series of cases, altho in morphology and in appearance of the growth on blood agar the difference is sharp.

³ Jour. Amer. Med. Assn., 1918, 71, p. 1733

TABLE 1
SHOWING THE CLOSE RELATIONSHIP BETWEEN THE DIFFERENT STRAINS

No. of Strains	Bile Solu- bility		Fermentation						
			Lactose		Inulin		Mannit		
	+	—	+	—	+	—	+	—	
108	0	108	105	3	4	104	5	103	Mathers' coccus
85	0	64	84	1	2	62	1	63	Streptococcus buccalis
27	27	0	27	0	22	5	22	5	Pneumococcus IV

TABLE 2
METHODS OF OBSERVATION EMPLOYED

Day of Disease	Temp.	Leuko- cyte Count	Pfeiffer Bacillus	Mathers' Coccus	Other Bacteria	Remarks
Case 21: (Influenza- Pneumonia)						
2.....	103.0	7,300	—	+	Pneumococcus IV M. catarrhalis	} M. catarrhalis more abundant than any other on these dates
3.....	102.0	5,500	—	+	M. catarrhalis	
4.....	103.0	5,000	—	—	M. catarrhalis	
5.....	103.0	3,700	—	—	Many diphtheroids	
7.....	104.2	4,600	—	—	Many staphylococci	} Almost pure culture of staphylococci in nasopharynx. This is rather unusual
8.....	104.0	4,200	—	—	Many diphtheroids and streptococci	
9.....	103.2	6,200	—	—	Many staphylococci	
15.....	100.4	9,900	+	—	Streptococcus buccalis, Pfeiffer bacillus very abundant	
18.....	99.4	14,400	+	+	Some diphtheroids	}
21.....	98.4	14,200	+	+	Strep. buccalis	
26.....	98.0	12,900	+	—	Strep. buccalis	
37.....	98.0	(24th) 7,800	—	—	Strep. buccalis M. catarrhalis	
Case 24: (Uncomplicated Influenza)						
1.....	102.0	—	—	—	Staphylococci	}
2.....	102.0	6,000	—	—	Diphtheroids	
3.....	100.6	5,400	—	—	Diphtheroids, M. catarrhalis	
5.....	99.0	—	+	—	M. catarrhalis	
6.....	99.0	8,000	+	—	B. mucosus capsulatus	}
8.....	Dis charged					
Case 66: (Uncomplicated Influenza)						
1.....	103.0	4,900	—	+	A few Strep. buccalis	} Almost pure culture Mathers' coccus Mathers' coccus not nearly so abundant as on preceding days
2.....	100.0	—	—	+	A few Strep. buccalis	
3.....	100.0	—	—	+	Staphylococci	
5.....	97.6	—	+	+	Many staphylococci	
7.....	97.0	7,460	+	+	Few if any bacteria besides Pfeiffer bacillus and Mathers' coccus	}
9.....	97.0	7,300	+	+	Mathers' coccus abundant	
14.....	Dis charged					

Attempts to differentiate the Mathers coccus and *S. buccalis* by testing their fermentation powers on a large number of carbohydrates have given negative results. A comparison of ten strains of each gave results as follows: Positive: lactose, saccharose, maltose (1 strain of *S. buccalis*, negative), galactose (1 *buccalis*, negative; same strain negative in maltose); mannose (2 *S. buccalis*, negative). Negative: inulin, mannite, arabinose, raffinose, sorbite and dulcitol. Type pneumococci (I, II and II) gave similar results with these carbohydrates except that inulin and mannite were fermented by all.

The methods of observation of the cases studied is illustrated in table 2.

In all, 47 cases of influenza were studied in this way. The distribution of the Pfeiffer bacillus and the Mathers coccus was as follows:

TABLE 3
DISTRIBUTION OF THE BACTERIA IN SAME EPIDEMIC

	No. Cases Exam- ined	No. in Which Pfeiffer Bacillus Was Found	No. in Which Mathers' Coccus Was Found
*Group A-1 Uncomplicated influenza (October).....	11	8	4
Group A-2 Influenza-Pneumonia (October).....	8	3	8
Group B Hospital influenza and influenza-pneumonia.....	17	11	8
Group C Uncomplicated influenza (December-February).....	11	8	11
	47	30 = 64%	31 = 66%

* These groups are described in the first paragraph in this paper.

Arrangement in such a numerical table has its limitations and does not give a complete picture of the findings since most of the cases in Groups A-1, A-2, and C were examined many times while nearly all of those in Group B were examined only once. Groups A-1 and A-2 are fairly comparable with one another in respect to the number of observations in each case, and so far as the examination of this limited number of cases from one localized epidemic is concerned, there is no doubt that Pfeiffer bacilli occurred more frequently and more abundantly in the uncomplicated influenza cases than in those in which pneumonia developed.

Comparison of the tabular record for Groups A-1 and C, on the other hand, might be misleading since the Pfeiffer bacillus was present in much larger numbers in the December-February cases (Group C) than in the October cases (Group A-1). Whereas, in the earlier cases Pfeiffer bacillus colonies were relatively infrequent compared with the numbers of other bacteria, in the later cases there were many plates in which the Pfeiffer bacillus and the Mathers coccus were practically the only organisms present. Considering the number of colonies

of Pfeiffer bacillus on each plate and the proportion of daily observations that were positive, the actual abundance of this organism in the upper respiratory tract was far greater in the later cases than in the cases observed during the height of the epidemic in Chicago. The contrast between the cases in Group B and Groups A-1 and A-2 (Table 3) was even more pronounced. Altho the total of examinations in the hospital cases was much smaller, the Pfeiffer bacillus was present in a relatively high proportion of the plates examined and usually in great numbers. The significance of relative abundance as disclosed by plate culture is somewhat problematical and the bearing of such facts on the rôle of the Pfeiffer bacillus must be regarded for the present as quite uncertain.

In 18 cases in which the Pfeiffer bacillus was found, examinations made on the 1st to 3rd day of the disease showed this organism present in 8, the Mathers coccus in 11 cases; in seven cases neither of these organisms were found on the first three days of the disease. In six of the ten cases in which the Pfeiffer bacillus was not found on the first three days, it was found later (See, for example, Table 2, Cases 24 and 66). The Mathers coccus, when present at all, was always found on the early days of the attack.

Other Bacteria.—The usual bacteria of the upper respiratory tract were found in most of the cases altho in greatly varying numbers, both in different individuals and in the same individuals on different days. The men in the Section B epidemic group (Table 1, Groups A-1 and A-2) harbored a far greater variety of bacteria than patients from other sources. The close contact of these men with one another during their preliminary illness and in the emergency hospital evidently favored a generous transfer of bacteria from throat to throat. The result was that at the time of our examination the variety of bacteria that had found a congenial soil was very large. Diphtheroids were especially numerous and were found richly in all the earlier cases. *M. catarrhalis* was also very commonly present, often in great numbers (See Table 2, Case 21). *Streptococcus buccalis* occurred in varying numbers, but was rarely very abundant. In many of the Section B cases in October a large gram-negative diplococcus was present which formed very delicate translucent colonies and died out readily even when transferred frequently on blood agar. It grew in the first generation very scantily on plain agar and since it did not ferment dextrose is perhaps to be regarded as belonging to the

M. catarrhalis group, altho under the conditions of our work it proved a much less vigorous organism than *M. catarrhalis* or than the Pfeiffer bacillus.

Pneumococci were found in ten cases, but several strains were irregular in respect to inulin and mannite fermentations. All but one (IIa) fell in Group IV on application of the agglutination test.

The Friedländer bacillus was found in four cases, in one of these in large numbers.

Hemolytic streptococci were found in seven cases, all but one of these (5) in the later stages of the epidemic; in five cases they were numerous. In one patient no hemolytic streptococci were observed during the primary attack (3 sets of examinations). Twelve days after recovery and discharge, the patient was readmitted with a temperature of 104 F. and subjective symptoms described as being very similar to those of the original attack; hemolytic streptococci were present in practically pure culture in throat and nasopharynx. This second attack or relapse was of short duration and the patient was discharged 5 days later. Bacteriologically it appeared like a new infection with an organism not originally present. The leukocyte count on the 4th day of the second attack was 14,500. A second case of the same nature was observed later.

Bacteriology of Colds, etc., During the Epidemic.—Twenty-eight cases of tonsillitis, sore throat and common cold among University students were examined, about half of these (13) while the influenza epidemic was at its height in October-November, 1918, the others in January-March, 1919, after the influenza cases had practically disappeared from the neighborhood. The organisms most commonly found on blood-agar plates were as follows:

Total cases examined.....	28
Mathers' coccus	15
Hemolytic streptococci	12
Pneumococcus IV	7
Pfeiffer bacillus	4

The Friedländer bacillus was found once in abundance and *M. catarrhalis* was found several times, though not in large numbers.

Observations on these cases were made in precisely the same manner as on the influenza cases. Typical records in Table 4 may be compared with the influenza records in Table 2.

TABLE 4
A SERIES OF TYPICAL RECORDS

Day of Disease	Temp.	Leuko- cyte Count	Pfeiffer Bacillus	Mathers' Coccus	Other Bacteria
Case 100 (Common Cold)					
2.....	104	8,900	—	—	<i>M. catarrhalis</i>
6.....	101.6	8,100	—	—	Hemolytic streptococcus
7.....	102	7,800	+	—	<i>Streptococcus buccalis</i>
Case 101 (Severe Cold, Sore Throat)					
3.....	102	10,500	—	—	<i>M. catarrhalis</i>
5.....	100	8,100	+	+	
6.....	99	7,900	+	+	<i>Pneumococcus IV</i>
7.....	...	6,000	—	+	
8.....	...	5,400	—	+	<i>Streptococcus buccalis</i>
13.....	100	10,200	—	—	<i>Streptococcus buccalis</i>
14.....	99	7,600	—	+	<i>Streptococcus buccalis</i>
Case 103					
1.....	98	12,900	—	+	<i>Pneumococcus IV</i> , <i>S. buccalis</i>
2.....	98	—	+	<i>Pneum. IV</i> , <i>S. buccalis</i> , <i>M. cat.</i>
4.....	98	10,300	—	+	<i>Pneum. IV</i> , <i>S. buccalis</i> , <i>M. cat.</i>
5.....	98	7,800	—	+	<i>S. buccalis</i> , <i>M. catarrhalis</i>
7.....	98	6,600	—	+	<i>Pneum. IV</i> , <i>S. buccalis</i> , <i>M. cat.</i>
Case 111* (Tonsillitis)					
2.....	102	9,700	—	+	Hemolytic streptococci (almost pure culture, very few other colonies)
4.....	...	13,400	—	+	<i>S. buccalis</i>

* Case 111 was one of four cases of tonsillitis developing at the same time, and all with a history of exposure at a party 18-24 hours before attack. All four cases showed leukocytosis during the first 4 days of the attack, the maximum ranging from 13,400 in one case to 20,800 in another. Three of the cases showed a relative increase of the neutrophils (82 to 86%). The bacteriologic picture as shown in throat and nasopharynx swabs was remarkably similar. Hemolytic streptococci were very abundant on all plates and were by far the predominating organisms. In one case pneumococcus (Type IV) was present in small numbers in one examination, but in the other only a few colonies of the Mathers streptococcus and of *S. buccalis* were found in addition to the hemolytic streptococci. Between the first and second examinations two throat treatments with methylene blue were given, but the character of the flora of nasopharynx and throat was not appreciably altered by this procedure.

The clinical picture of these common endemic respiratory tract infections was distinctly different from that of epidemic influenza altho many of the symptoms were similar and the attack at first was frequently regarded by the patient and others as influenza. Headache, dizziness and pain in the joints and limbs were common accompaniments of these cases and in some instances the onset was sudden. On the other hand, sore throat was much more frequent and pronounced than in the cases of epidemic influenza and patches were often observed on the mucous membrane. In the majority of cases the temperature did not run above 102 F. and the fever was not prolonged beyond 24-48 hours. The leukocyte count was high in nearly all these cases. Thirty-nine leukocyte counts made in 20 cases, usually on the

2 days of the height of the attack gave an average of 11,500. The range was as follows:

Above 10,000	22
7,000-10,000	11
Below 7,000	6

In only one case was the leukocyte count similar to that observed in the majority of cases of true influenza. In this case (107) the leukocytes were: 3rd day, 6,700; 4th, 4,500; 5th, 5,200; 7th, 5,100. The symptoms were not unambiguous: headache, pain in back, no sore throat, no cough, no nosebleed. The temperature was not high (2nd day, 101.8) and quickly subsided (3rd day, 98.6) and none of the symptoms were severe. The predominating organism on the 3rd, 4th and 5th day was the Mathers coccus altho some Pneumococci (Type IV) were found on the 4th and 5th days. On the 7th day many Friedländer bacilli were found. This case may have been one of light influenza, but there were no other evident cases in the neighborhood or among the associates of (107) at this time (Feb. 2-8, 1919).

In comparing the October-November cases of cold with those in January-March the most conspicuous difference was the much larger number of cases with hemolytic streptococci and with pneumococci (Type IV) in the latter group.

TABLE 5
COMPARISONS MADE AT DIFFERENT PERIODS DURING THE EPIDEMIC

	Height of Influenza Epidemic Oct.-Nov., 1918	After Subsidence of Influenza Epidemic Jan.-March, 1919
No. cases.....	13	15
Hemolytic streptococci.....	2	10
Pneumococcus IV.....	1	6
Mathers coccus.....	4	11
Pfeiffer bacillus.....	1	3

The richer flora in the second group of cases may be connected with the greater severity of these cases and this in turn, dependent on the season of year. At all events the relatively mild cases of common respiratory tract infection that were examined during the height of the influenza epidemic did not harbor the Pfeiffer bacillus in nearly as high proportion as did the influenza cases examined at the same time; neither did they, except in a few cases, harbor in abundance hemolytic streptococci or the Mathers' coccus. In several cases the only organ-

ism growing in any considerable numbers on blood-agar plates was the common mouth streptococcus (*S. buccalis*).

The association of large numbers of hemolytic streptococci (var. beta) with cases of tonsillitis and generally with cases of severe throat inflammation was markedly evident in this series. This corresponds with the relative scarcity of this organism in the cases of true influenza in which, as a rule, sore throat was not observed.

Leukocyte Counts.—In the course of these observations leukocyte counts were made as a rule on the same days when throat swabs were taken. The average counts in simple influenza showed a leukopenia as recorded in Table 6. The term simple influenza is here used to designate those cases not showing signs of clinical pneumonia. The patients usually regained a normal condition by the end of the first week, although with the ordinary aftermath of weakness.

TABLE 6
LEUKOCYTE COUNTS: SIMPLE INFLUENZA

Days	Cases	Total Leuko- cytes	Polymorpho- nuclears, per Cent.	Lympho- cytes, per Cent.	Large Mono- nuclears and Transitionals, per Cent.
2 and 3.....	13	6,100	63	25	9
4 and 5.....	9	4,900	62	33	5
6 and 7.....	6	6,100	59	32	9
8-12.....	10	7,500	66	25	9
12-30 (after full recovery and discharge)....	8	9,100	60	34	6

Eosinophils were generally absent during the attack.

Individual cases sometimes showed considerable deviation from the averages. In one case the lowest count was not reached until the 8th day of the disease (4,300). Several cases without any apparent complication gave leukocyte counts of over 10,000 for a week or more after seemingly complete recovery. In general, the counts ran rather high for some time after the patient was able to return to his ordinary avocation.

Similar observations on the influenza cases in which clinical pneumonia developed showed a drop in the number of leukocytes similar to that recorded above, but after a longer or shorter interval this was followed in each of the cases here observed by a moderate leukocytosis (14,000-15,000) which, however, in one case (Cf. 21, Table 2) did not appear until about the 15th day. The differential ratio was not appreci-

ably altered in these cases. Quite different is the normal leukocyte count in ordinary colds, tonsillar inflammation, etc.

TABLE 7
LEUKOCYTE COUNTS: COLDS, ETC.

Days	Cases	Total Leuko- cytes	Polymorpho- nuclears, per Cent.	Lympho- cytes, per Cent.	Large Mono- nuclears and Transitionals, per Cent.
1, 2 and 3.....	22	12,600	73	22	5
4 and 5.....	8	11,600	74	20	6
6 and 7.....	7	8,800	69	25	6
8-14.....	3	7,200	68	25	7

Comparison with Table 6 brings out very plainly the average differences in total leukocyte counts. Whether the slight divergences in the differential count that appear in these figures have significance will need a larger number of observations to determine. As already stated several cases have been observed in the course of this study which clinically seemed more like colds than like true influenza, but which had a low leukocyte count throughout. In the absence of any definite diagnostic criterion the relationship of these infections to true influenza must remain uncertain.

SUMMARY

The bacteriologic picture in influenza is not a uniform one so far as nose and throat flora is concerned. The ordinary methods of cultivation with blood-agar plates show marked differences in individual cases. Groups of individuals who have been in more or less intimate contact with one another may harbor very similar assemblages of micro-organisms, but differ from other groups examined at the same time. The variations in respiratory tract flora reported by many observers during the progress of an influenza epidemic are doubtless, in part, group differences.

Daily examination of a number of selected typical cases, mild and severe, have shown no one organism constantly demonstrable in large numbers by the methods employed. The two organisms most commonly and abundantly present in this series were the Pfeiffer bacillus and the diplococcus or streptococcus found by Mathers at Camp Meade.*

* This is apparently very similar to, if not identical with, the organism described by Zingher (Jour. Amer. Med. Assn., 1919, 72, p. 1020).

The Pfeiffer bacillus was found in 64% of the influenza cases examined between October, 1918, and February, 1919. It was present in much larger numbers in the throats of the patients examined toward the end of the outbreak. Several cases did not come under observation until clinical pneumonia had developed, and in these cases the Pfeiffer bacillus was not found as frequently as in the "uncomplicated" cases. Other cases for various reasons could be examined only once or twice, and the findings are not strictly comparable with those in which daily examinations were carried out. The percentage of positive findings would probably have increased if all patients had been subjected to examination throughout the course of the attack. The relative abundance of the Pfeiffer bacillus varied greatly. In some cases it was the predominant organism, in others only a few colonies could be found, no more than in normal throats. It did not often happen in this series that hemolytic streptococci (var. beta) and the Pfeiffer bacillus were both present in large numbers. On the other hand, the Pfeiffer bacillus and the Mathers coccus often had the field almost to themselves. In a few cases the Pfeiffer bacillus was present in such overwhelming numbers in cultures from nose, nasopharynx and throat that participation in a pathologic process was strongly suggested. These cases, however, did not differ clinically in any appreciable way from other cases in which the Pfeiffer bacillus was found scantily.

The Mathers coccus was found about as frequently and abundantly as the Pfeiffer bacillus, altho its occurrence was quite independent of that of the latter. Its association with the pneumonia cases seemed to be closer than that of the Pfeiffer bacillus, but it was also found in all the later cases of simple influenza. Variations in the abundance of this organism were quite as marked as were those of the Pfeiffer bacillus, and no relation could be demonstrated between these findings and the characters of the cases. Practically pure cultures of the Mathers coccus were obtained from the nasopharynx of some patients.

Comparison of the true influenza cases with colds and tonsillar infections showed that the general leukopenia of the influenza cases could be contrasted with the general leukocytosis of the others. Blood counts made during the first 4 or 5 days of the attack practically invariably showed at some time a leukopenia in the influenza cases and a leukocytosis in the others. The chief differences in the bacterial findings were the relative infrequency of the Pfeiffer bacillus in the colds, etc. (14%), and the relatively high proportion of hemolytic

streptococci (var. beta). The Mathers coccus was present in about the same proportion of cases as in influenza.

The pneumococcus was found in about 20% of the influenza cases (10:47), and in a slightly larger proportion in the cases of rhinitis and tonsillitis (7:28). No special search was made for these organisms, and it is probable that positive findings would have been increased somewhat if mice inoculation could have been made whenever throat swabs were taken. Washed sputum, when procurable, was inoculated into mice, but the pneumococcus was not invariably isolated. In two cases as much as 2 c.c. of washed sputum from influenza patients injected intraperitoneally did not lead to the death of the mouse. The pneumococci isolated from both influenza and rhinitis cases were all Type IV except one strain (IIa).

M. catarrhalis, the Friedländer bacillus and an unidentified gram-negative diplococcus were found, at times, in large numbers in the throat, nose and nasopharynx of influenza cases. Staphylococci were also sometimes present in great abundance.

Two observations on suspected cases of "recurrence" or "second attack" have shown the presence in the throat of organisms (hemolytic streptococci) not found during the original attack. Both recurrences presented some of the clinical symptoms of influenza, but had a moderate leukocytosis. A third case of suspected "recurrence" likewise showed hemolytic streptococci in the throat and slight leukocytosis, but this patient had not been under our observation during the primary attack. It seems probable that an alleged "second attack" of influenza occurring within a few weeks of the original attack is at least in some cases a new infection with another organism.

The observations carried out by the aerobic blood-agar plate method and recorded in this paper have not shown the predominance or constant presence of any one organism in the upper respiratory tract of influenza patients. The Pfeiffer bacillus, however, has been more conspicuous than any other organism, particularly in comparison with its relative infrequency in cases of rhinitis and tonsillitis examined during the same epidemic period.

THE INFLUENCE OF THE SURFACE TENSION OF THE CULTURE MEDIUM ON THE GROWTH OF BACTERIA *

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There is probably no phase of bacteriologic research that has occupied the attention of investigators more than the preparation of culture mediums. Most of such investigations have been directed toward supplying bacteria with the nutritive material best adapted to their specific individual requirements. Aside from its reaction, the physico-chemical state of the medium has received but little attention.

It is the purpose of this paper to record the behavior of some of the common bacteria when cultivated on medium of low surface tension.

Our investigation naturally began with a search for suitable substances with which to lower the surface tension of mediums. It was soon found that substances that would lower the surface tension of water would not always act satisfactorily in lowering the tension of broth or other mediums. Furthermore, only such surface tension depressants could be used as were of themselves toxic neither to bacteria nor to laboratory animals into which cultures or filtrates were subsequently to be injected. It was found that soap fulfilled our requirements better than anything else tried. A large variety of commercial soaps were used, after first being purified and neutralized. They all possessed certain disadvantages, however, as they invariably caused a marked turbidity of the medium. Furthermore, the surface tension of the medium depressed by the various soaps would not remain constant, due to the fact that soap tends to hydrolyze when diluted, which causes the tension to rise. Studies were therefore made in an effort to secure a soap that would be perfectly clear when in solution, and which would be sufficiently stable to prevent the objectionable fluctuations in the surface tension of the medium. It was found that pure castor oil saponified with NaOH met these requirements.

A number of neutralized fatty acids form a clear solution when hot, but become turbid and viscous on cooling. Solutions of castor oil soap

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on the other hand remain perfectly clear when cooled, do not hydrolyze on sterilization in flowing steam, nor do they become viscous when cooled.

Castor oil soap is prepared by adding an alcoholic solution of NaOH to castor oil slightly in excess of its saponification equivalent. The mixture is then heated over the water-bath until saponification is complete when the soap is dissolved in hot absolute alcohol to which is added a few drops of phenolphthalein. Hydrochloric acid, diluted with alcohol, is then added drop by drop until the soap solution is neutral. It is then filtered on paper to remove the chlorids. The excess of alcohol may then be removed by evaporation, after which the soap solution is poured into a saturated solution of NaCl to remove the glycerol and other impurities. After further filtration followed by washing with a saturated solution of sodium chlorid the soap is ready for use.

The drop method was used in measuring the surface tension.

$$\frac{\text{Weight of number drops of medium}}{\text{Weight of number drops of water}} \times 73$$

In order to give a general idea of the comparative surface tension of various fluids it may be stated that that of water is 73, alcohol 22, and ether 16 dynes.

The broth prepared in our laboratory by standard methods has a surface tension of about 59 dynes. This may vary slightly, but it was always found to be between 58 and 60 dynes, 59 being the value usually obtained. The surface tension of broth may be lowered to any desired point down to 32 dynes by adding the necessary quantity of soap solution to the medium.

In growing bacteria on broth of lower surface tension we were impressed by the behavior of the *B. subtilis* when the tension had been depressed below 45 dynes. When grown on such medium this organism no longer forms a pellicle, but grows down in the body of the medium. When the tension of the broth is depressed to below 45 dynes the growth of the *B. subtilis* is largely at the bottom of the tube. It was further observed that spore formation was markedly suppressed and sometimes entirely absent, especially after cultivation on low tension mediums for several generations. *B. subtilis* grown on medium of low surface tension may sometimes be rendered sterile by heating to 60 degrees for an hour which indicates that spore formation has been entirely suppressed. It is generally supposed that *B. subtilis* grows on the surface of broth because of its great demand for oxygen.

Since our work has shown that this organism is capable of growing throughout the medium, even at the bottom of the tube, it is evident that its oxygen requirement is not the chief reason for surface growth on ordinary broth. In this connection we would not overlook the fact that lowering the surface tension of water increases its ability to dissolve oxygen and other gases. A striking feature common to most bacteria which grow on the surface of liquid mediums, particularly *B. subtilis*, *B. mesentericus*, and *B. tuberculosis* and others, is that whenever there is pellicle formation there is little or no growth except at the very surface. In inoculating a flask of glycerol broth with *B. tuberculosis* large numbers of organisms may sink to the bottom of the flask, but only those that remain at the surface show signs of multiplying. Other bacteria, such as *B. coli*, the cholera spirillum, first show signs of development at or near the surface even though there is no pellicle formation. What can be the explanation of this selective site of so many bacteria? It is possible that at the surface of the medium certain substances are concentrated which favor, or are even necessary, for the metabolism of many micro-organisms. It is a well known fact that surface energy tends to reduce itself to a minimum, and that this may be accomplished in two ways: (1) by the reduction of the surface area of the liquid, or (2) by reducing the surface tension, or both. The surface tension of the liquid is reduced to the lowest point possible through the concentration of the surface tension depressants at the surface of the liquid. *B. subtilis* and other bacteria, which show such predilection for the surface of the medium, probably select this zone because the surface tension reducing substances concentrated there are required for their metabolism. The pellicle formation is probably a mere coincident, the surface tension of the fluid being sufficient to support the weight of the organisms just as certain insects may be supported on the surface of water because of its high surface tension.

The question naturally suggests itself as to whether or not all aerobic bacteria might not become pellicle formers were the surface tension of the culture medium sufficiently high. Since it is impossible to raise the tension of a medium much above that of water, this experiment has its limitations. However, we have found that *B. coli* as well as some of the paratyphoid bacteria form well developed pellicles when grown on an inorganic medium having a surface tension of 72.2 dynes.

Investigations have also been conducted with reference to the influence of the surface tension of the medium on some of the common anaerobic bacteria, such as *B. tetani*, *B. botulinus*, *B. chauvei*. It was found that *B. tetani* grew well under ordinary aerobic conditions providing the surface tension of the medium had been properly depressed. *B. tetani* produces a potent toxin when grown on ordinary broth, the surface tension of which has been reduced to about 40 dynes.

This would seem to indicate that we cannot draw a sharp line between the aerobic and anaerobic bacteria although there can be no question but that some bacteria demand a liberal supply of oxygen, while others cannot thrive where the supply is too liberal. In this connection it may be pointed out that one of the popular methods of producing anaerobiosis is to cover the medium with a layer of sterile oil, the object of the oil being to exclude oxygen from the medium which it covers.

Since oil is a much better solvent of oxygen than water, is it reasonable to suppose that oil actually does exclude oxygen? If the tube were sealed off immediately after introducing oil into the test tube it might be argued that the oil, because of its being a better oxygen solvent than the medium, would cause the gas to diffuse from the medium into the oil later; but since the sealing of the tube following the introduction of the oil is not necessary to effect the so-called anaerobic condition, this is obviously not the explanation. A little experiment which shows that the oil does not exclude oxygen from culture medium may be made by inoculating a tube of broth with a culture of *B. subtilis* after which the inoculated medium may be covered with a layer of sterile oil; 48 hours following this inoculation it will be found that the *subtilis* has formed a very heavy pellicle just beneath the oil layer, showing that oxygen does pass from the oil into the medium, otherwise this organism would not be able to grow at this point. The oil undoubtedly favors the growth of anaerobic bacteria because it reduces the surface tension of the medium, and as a result the surface tension depressants contained in the medium are not concentrated at the surface as would ordinarily be the case, but are dispersed throughout the medium where they can serve the metabolic requirements of the anaerobic bacteria that under ordinary circumstances would be unable to avail themselves of these surface tension

lowering substances because of the excess of oxygen at the surface where they are concentrated.

The mere act of lowering the surface tension of the medium will not cause all anaerobes to grow under ordinary aerobic conditions. Such a germ as *B. chauvei* fails to show any appreciable development under such conditions.

Most bacteria seem to grow best when the surface tension has not been depressed below 53 to 55 dynes, although most of the more common bacteria, such as the staphylococcus, *B. coli*, *B. typhosus*, and *B. paratyphosus* show some growth when the surface tension has been depressed as low as 32 dynes.

Surface tension below 45 dynes tends to cause many bacteria to agglutinate, thus giving the impression that the growth is entirely at the bottom of the test tube, this being particularly true of the typhoid and paratyphoid bacilli. The streptococci and pneumococci do not grow well where the surface tension has been brought below 50 dynes. The pneumococci show a tendency to disintegrate when grown on a medium of a low surface tension. This observation suggests that the solution of pneumococci by bile may be due, in part at least, to the lowering of the surface tension which bile induces. *B. tuberculosis* grows very well on medium of low surface tension. Like *B. subtilis* it no longer grows with pellicle formation, but grows at the bottom of the medium.

The pathogenicity of bacteria seems to be profoundly affected by the surface tension of the medium on which they are grown. We hope to give a detailed report on the relation of pathogenicity and toxin formation of bacteria to the surface tension of the medium in a subsequent article.

SUMMARY

The growth of bacteria on ordinary broth is greatly influenced by the surface tension of the medium.

A sodium soap of castor oil proved to be a practical surface tension depressant.

All pellicle formers cease to grow at the surface where the tension is below 45 dynes.

The tendency of *B. subtilis* to form spores is considerably reduced when the organism is grown on broth medium of low surface tension.

Some anaerobes, *B. tetani* in particular, grow well under ordinary aerobic conditions on medium of reduced surface tension. The layer of oil often used to cover mediums for the purpose of producing anaerobiosis, probably acts by reducing the surface tension rather than by excluding oxygen.

CLASSIFICATION OF STREPTOCOCCI BASED ON THE CORRELATION OF RESULTS OF HEMOLYTIC, FERMENTATIVE AND PRECIPITIN TESTS

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In the literature correlation of the hemolytic, fermentative, and serological reactions of streptococci has yielded little of practical value. In the hands of different investigators similar strains have produced varying correlated results.

HEMOLYTIC REACTIONS

Since the discovery of streptococcus hemolysins by Marmorek¹ in 1902, many questions bearing on this phase of streptococci have been investigated and hemolysis has been made a basis for their classification by Schotmüller.² The blood of many species of animals has been tried.³ In general three preparations of blood have been used, namely, unwashed defibrinated blood,⁴ washed red blood cells, and a combination of fresh blood with various mediums. Besredka⁵ was one of the early workers to investigate the hemolytic effects of streptococci, and experiments with the blood of man, goat, sheep, goose, and chicken, obtaining no results with the last two. M'Leod⁶ established a minimum hemolytic dose with washed red blood cells of the ox. Lyall⁷ classified streptococci according to their metabolic gradients and correlated the results with their hemolytic effect on washed red blood cells of the sheep. He showed the advantage of using constant amounts of the washed corpuscles (a 5% suspension) over that of blood agar. Blake⁸ showed that *S. viridans*, considered nonhemolytic by Holman,⁷ produces methemoglobin by adding equal amounts of the culture grown in ascitic broth to a 5% suspension of washed sheep corpuscles.

The most common method of demonstrating the hemolytic properties of streptococci is to cultivate them on nutrient agar to which has been added a small quantity (5%) of freshly drawn blood, as shown by Baerthlein,⁸ Becker,⁹ Benthin,¹⁰ Cole,¹¹ Conrad,¹² Davis,¹³ Levy,¹⁴ Rosenow,¹⁵ and Van

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¹ Ann. de l'Inst. Pasteur, 1902, 16, p. 172.

² München. med. Wchnschr., 1903, 20, p. 894.

³ Ann. de l'Inst. Pasteur, 1901, 15, p. 880.

⁴ Jour. Path. and Bacteriol., 1911, 12, 16, p. 321.

⁵ Jour. Med. Research, 1914, 30, p. 515 and p. 487.

⁶ Jour. Exper. Med., 1916, 24, p. 4.

⁷ Jour. Med. Research, 1916, 34, p. 377.

⁸ Centralbl. f. Bakteriöl., I. O., 1914, 74, p. 201.

⁹ Jour. Infect. Dis., 1916, 19, p. 754.

¹⁰ Centralbl. Bakteriöl. I. O., 1912, 67, 1883, p. 92.

¹¹ Jour. Exper. Med., 1914, 20, p. 363.

¹² Beitr. z. Geburtsh. u. Gynäk., 1909, 13, p. 364.

¹³ Jour. Infect. Dis., 1917, 21, p. 308.

¹⁴ Virchows Archiv, 1907, 187, p. 327.

¹⁵ Jour. Am. Med. Assn., 1914, 14, p. 1.

Logham,¹⁶ Schotmüller,² using human blood agar, divided streptococci into three classes: those giving a clear colorless zone around the colonies—*S. pyogenes*; those producing a green colony—*S. mitior* seu *viridans*, and small mucoid colonies with a dark green zone around the colonies in hemoglobin medium—*S. mucosus*. Libman¹⁷ described a series of concentric hemolytic zones from the action of streptococci on blood agar, and Ruediger¹⁸ discussed the changes produced on blood in blood agar by bacteria. He decided that the green coloration was due to lactic acid in combination with blood and glucose. Benthin,¹⁰ using 13 strains of streptococci, showed that glucose added to blood agar inhibited hemolysis. Braun¹⁹ determined hemolysin producers by growing streptococci on rabbit blood agar. Van Logham,¹⁶ using *V. El Tor* and *Vibrio cholerae*, differentiated between hemolysis as shown by a bright halo around colonies on blood agar plates, and hemodigestion shown by a green coloration around the colonies on the same plate. Baerthlein⁸ described three different changes by bacteria: (1) simple hemolysis in which the hemoglobin is extracted leaving the stroma preserved, occurring in fluid medium; (2) hemoglobin digestion in which blood pigments are completely destroyed with stroma unaffected, and (3) hemodigestion in which both pigment and stroma are destroyed. Rosenow²⁰ claimed to have changed the hemolytic and virulent properties of streptococci by growing them symbiotically with *B. subtilis* on blood agar. Becker⁹ recognized the necessity of a standardized grade of blood agar and found that hemolysis was inhibited by dextrose. Davis,²³ on the other hand, claimed that dextrose did not hinder hemolysis. His observation thus differed from that of Benthin's and Becker's. This was probably due to the fact that Benthin and Becker failed to differentiate between the action of hemolysins and that of acid on the mediums.

VIRULENCE IN RELATION TO HEMOLYSIS

Marmorek¹ first called attention to a possible relation between hemolytic activity and virulence by stating that the more virulent the strain the more quickly it dissolves the red blood corpuscles in the host. Schotmüller,² making his classification of streptococci on their hemolytic effects, did not believe, however, that the virulence of certain strains was related to their hemolytic reactions. Likewise de Waele and Sugg²¹ concluded that the hemolytic reactions of streptococci are not proportional to their virulence. Fromme,²² working with streptococci isolated from the peritoneum after laparotomy, assumed a neutral attitude as to relation of hemolysis to pathogenesis. Zangmeister,²³ growing streptococci on human blood agar plates, decided that there is no relation between hemolytic actions and virulence; however, after examining a large number of cases, he observed that 73% of puerperal women harboring hemolytic streptococci gave a temperature reaction, while of those harboring nonhemolytic streptococci only 12% showed rise in temperature. Braun¹⁹ obtained varying results depending on the incubation period of the organisms, the amount of inoculum, and the animals infected. M'Leod²⁴ found that by

¹⁶ Centralbl. f. Bakteriöl. I, O., 1913, 70, p. 70.

¹⁷ Proc. N. Y. Path. Soc., 1905, v, p. 59.

¹⁸ Jour. Infect. Dis., 1906, 3, p. 663; 3, p. 755.

¹⁹ Centralbl. f. Bakteriöl. I, O., 1912, 62, p. 383.

²⁰ N. Y. Med. Jour., 1914, 99, p. 270. Jour. Infect. Dis., 1914, 14, p. 1.

²¹ Centralbl. f. Bakteriöl. I, O., 1905, 39, p. 324.

²² Arch. f. Gynäk., 1908, 32, p. 1213.

²³ Deutsch. med. Wchnschr., 1909, 35, p. 427; 1910, 37, p. 1268.

²⁴ Jour. Path. and Bacteriol., 1914-15, 19, p. 392; 1911-12, 16, p. 321.

using constant methods the degree of hemolysis of streptococci in vivo was related to virulence but that the results were not as constant as when the organisms were grown on blood agar. Davis²⁵ concluded that since hemolytic streptococci are so prone to attack serous surfaces they should be suspected as virulent agents in the causation of disease. Lyall⁵ observed a definite relationship between hemolysis and virulence, and he believes that there is a relation between their solubility in bile and their virulence. Rosenow²⁰ observed a direct relationship between hemolytic streptococci and acute streptococcal infections, while chronic streptococcal infections were more closely associated with the viridans type. Holman⁷ believes that, since the majority of streptococci producing pyogenic infections are hemolytic and those of a nonhemolytic power produce less acute diseases, the property of hemolysis in relation to virulence should be recognized.

FERMENTATIVE REACTIONS

Winslow,²⁷ classifying streptococci according to their action on carbohydrates, suggested the possibility of a metabolic gradient. He showed that there were three specific strains: those fermenting monosaccharids only; those fermenting monosaccharids and disaccharids, and those fermenting monosaccharids, disaccharids and trisaccharids. Walker²⁸ found that on cultivation and inoculation in white mice, streptococci gave varying fermentative reactions in the same sugars. Holman²⁹ obtained specific and constant results with Hiss serum water medium and Andrade's indicator. He used lactose—a disaccharid; mannite—an alcohol, and salicin—a glucosid. Thro,³⁰ using dextrin, arabinose, mannite, salacin, raffinose, lactose, saccharose and inulin with litmus indicator, failed to secure uniform results.

IMMUNOLOGICAL REACTIONS

Kinsella and Swift,³¹ examining nonhemolytic streptococci for specificity of complement fixation, obtained overlapping but apparently related results. In some reactions the antigen was specific for one serum while in others the serum was fixed by several antigens. They explain their results by saying that some streptococci have a more complex chemical nucleus than others. Howell,³² working with strains of nonhemolytic streptococci from specific disease finds that there is a tendency for them to react alike in complement fixation. Aschner³³ obtained marked fixation with the homologous antigen and a positive but less marked fixation with mixed antigen.

Since Bordet observed agglutination of cholera vibrios by immune serum, many have tried to correlate the various cultural relationships of streptococci with agglutination (Besredka,³⁴ Dick,³⁵ Floyd and Wolbach,³⁶ Hiss,³⁷ Kligler,³⁸

²⁵ Arch. Int. Med., 1912, 9, p. 505.

²⁶ N. Y. Med. Jour., 1914, 99, p. 270, and Jour. Infect. Dis., 1914, 14, p. 1.

²⁷ Jour. Infect. Dis., 1912, 10, p. 285.

²⁸ Proc. Roy. Soc., B., 1912, 85, p. 400.

²⁹ Jour. Infect. Dis., 1914, 15, p. 208; Jour. Med. Research, 1916, 34, p. 377.

³⁰ Jour. Infect. Dis., 1914, 15, p. 234.

³¹ Jour. Exper. Med., 1917, 25, p. 877.

³² Jour. Infect. Dis., 1918, 22, p. 230.

³³ Jour. Infect. Dis., 1917, 21, p. 409.

³⁴ Ann. de l'Inst. Pasteur, 1904, 18, p. 363, and p. 373.

³⁵ Jour. Infect. Dis., 1913, 12, p. 111.

³⁶ Jour. Med. Research, 1913, 14, 29, p. 493.

³⁷ Jour. Exper. Med., 1905, 7, p. 547.

³⁸ Jour. Infect. Dis., 1915, 16, p. 327.

Krumwiede and Valentine^{38a}), but with no marked success. Swift and Thro³⁹ obtained specific reactions for group classification of streptococci, but not for specific strains, by complement fixation and agglutination. Wolbach⁴⁰ comparing his agglutination and fermentation reactions obtained promising results which, although not specific enough for a group classification, warranted the belief that streptococci might be classified into fewer groups.

My own work shows the feasibility of employing the precipitin reaction successfully in the grouping of streptococci and that the serological distinctions are correlated with cultural differences. Kraus⁴¹ showed that when the germ-free filtrates of cultures of cholera, typhoid, and plague bacilli are mixed with their respective serums, a precipitate is formed. Tchistowitch⁴² speaks of Marmorek as the first to report a streptococcic precipitin. Gay and Chickering⁴³ made use of the precipitin reaction in the concentration of the protective bodies in antipneumococcus serum. Blake,⁴⁴ by a simpler method for obtaining precipitinogen, established an efficient precipitin test for the diagnosis of different types of pneumonia.

METHODS USED IN STUDY OF HEMOLYSIS AND FERMENTATION OF STREPTOCOCCI

The purpose of this investigation was to attempt to correlate if possible the hemolytic, fermentative, and precipitin reaction of certain strains of streptococci. Fifty-one strains were chosen, isolated from various sources as follows: 18 from sputum; 4 from infected tonsils; 3 from pyorrhea; 1 from empyema; 1 from gastric ulcer; 1 from the heart blood in a case of nephritis; 1 from a blood culture in septicemia; 1 from heart blood in leukemia; 1 from feces; 7 from urethral discharges; 1 from urine; 2 from blood cultures in cases of epilepsy; 1 from cervix of uterus; 5 from nose and throat swabs in cases of colds; 2 from sources unknown; 1 from abscess in guinea-pig, and 1 from an infection in a horse, disease unknown.

These cultures were examined for hemolysis simultaneously by three different methods: by inoculation into a 5% suspension of rabbit corpuscles; by growing them on rabbit blood sugar-free agar, and by growing them on rabbit blood nonfermented agar. The mediums were made as follows:

1. Rabbit Blood Sugar-Free Agar: Ground bob veal, 500 gm.; tap water, 700 c c; boiled 5 minutes; strain through a tin strainer; rinse with tap water q. s., to 1,000 c c; transfer to a clean kettle and boil; strain through cheese cloth; adjust to +1.0; sterilize in Erlenmeyer flasks at 100 C. for 2 hours;

^{38a} Jour. Infect. Dis., 1916, 19, p. 760.

³⁹ Arch. Int. Med., 1911, 7, p. 24.

⁴⁰ Jour. Med. Research, 1914, 29, p. 493.

⁴¹ Wien. klin. Wchnschr., 1897, 10, p. 736.

⁴² Ann. de l'Inst. Pasteur, 1899, 13, p. 406.

⁴³ Jour. Exper. Med., 1915, 21, 4, p. 389.

⁴⁴ Ibid., 1917, 36, p. 67.

inoculate with *B. coli communis* and incubate 24 hours at 37 C.; transfer to a suitable pan and boil 10 minutes; restore to original quantity by adding distilled water. Add: peptone, 1%; NaCl, 0.5%. Add: Japanese agar, 3%; readjust to +1.0 phenolphthalein; sterilize in Erlenmeyer flasks at 10 lbs. for 1 hour; cool to 45-42 C. Add: 7.2% sterile rabbit blood; tube or draw into petri dishes sterily; incubate 24 hours for contamination tests.

2. Rabbit Blood Nonfermented Agar: Bob veal, 500 gm.; tap water, 700 cc; boil 5 minutes; strain through a tin strainer; rinse with tap water q. s., to 100 cc; transfer to a clean kettle. Add: peptone (Digestive Ferments Co.), 1%; NaCl, 0.5%; titrate and adjust to +1.0%. Add: dissolved Japanese agar, 3%; readjust to +1.0; sterilize 1 hour at 10 lbs. Add: sterile rabbit blood, 7.5%; tube about 10 cc per tube; incubate the tubes for 24 hours at 37 C.

TABLE 1
STREPTOCOCCI STUDIED, CLASSIFIED ACCORDING TO HEMOLYSIS AND FERMENTATION

Hemolytic Reaction	Classification of Cultures by Hemolytic and Fermentation Tests	Number of Cultures	Source of Cultures
Hemolytic	<i>S. infrequens</i>	6	Sputum
		1	Gastric ulcer
		3	Tonsils
		1	Heart blood in nephritis; necropsy case
		4	Urethra
		1	Empyema
		1	Cervix uterus
		1	Nasal swab
		1	Guinea-pig, infected joint
		1	Source unknown
	<i>S. pyogenes</i>	1	Tonsils
		1	Urethra
		1	Gums in pyorrhea
		1	Sputum
		1	Blood culture; epilepsy
	<i>S. anginosus</i>	1	Epilepsy, throat swab
		5	Sputum
	<i>S. subacidus</i>	1	Septicemia blood culture
		2	Sputum
		1	Empyema pus
		1	Veterinary; source not known
	<i>S. hemolyticus</i> No. 2	1	Throat swab
Nonhemolytic	<i>S. fecalis</i>	1	Throat swab
		1	Sputum
	<i>S. mitis</i>	2	Sputum
		1	Human feces
		1	Leukemia; heart blood; necropsy case
	<i>S. nonhemolyticus</i> No. 2	1	Pyorrhea
	<i>S. ignavus</i>	1	Unknown
		1	Pyorrhea
	<i>S. equinus</i>	3	Urethral
	<i>S. salivarius</i>	1	Throat swab
		1	Nasal swab

The blood agar was drawn into petri dishes. The stock cultures were maintained on rabbit blood nonfermented agar, and in tubes containing 1 cc of defibrinated blood. Cultures, when properly sealed,

may be kept alive from 10-12 months or even longer, in defibrinated blood at room temperature.

Table 1 shows the classification of the streptococci based on hemolysis in the 5% suspension of rabbit corpuscles and on a modification of Holman's⁴⁵ fermentative mediums, described later. It was noted that the incubation of streptococci in a suspension of red blood cells was a more delicate method of testing hemolysis than growth on blood agar. The readings for hemolysis in table 1 represent observations made at the end of 4 hours' incubation, observations being made every 15 minutes beginning at the end of the first 10 minutes. The rapidity with which hemolysis took place varied with different cultures.

Hemolytic streptococci gave three different and distinct changes in the washed corpuscles. Some of them produced a laking of the corpuscles without digestion of the stroma; another series laked the corpuscles and then changed the hemoglobin to a brown color (methemoglobin) without digestion of the stroma, while the third attacked the corpuscles so violently that there was no apparent intermediary stage, that is, laking of blood, but an oxidation to methemoglobin with the digestion of the stroma of the cell as explained by Ruediger.⁴⁶ These when grown on blood agar produced a green pigmentation and would thus be classed as *Strep. viridans* (nonhemolyzers).

A comparison of the hemolytic reactions on the sugar-free and the regular unfermented blood agar showed no wide differentiation. Tests were made with the hemolytic strains to determine the effect of dextrose on their hemolytic powers in blood agar. Blood agar containing 0.5, 1, 1.5 and 2% glucose was used. No hemolytic inhibition could be observed by the cultures tried until an addition of over 1% dextrose was used.

Increasing the glucose content of the blood agar medium increases the amount of green pigment produced by the viridans group of streptococci. This observation has been reported by Ruediger.⁴⁶ Some of the nongreen producers on sugar-free blood agar when grown on a 1 or 2% dextrose blood agar produced a green pigment—a fact which agrees with Crowe's⁴⁷ and Sullivan's⁴⁸ observations.

Three tests of the fermentative reactions were carried out at intervals of 3 months with constant results. Extreme care was taken to

⁴⁵ Jour. Med. Research, 1916, 34, p. 377.

⁴⁶ Jour. Am. Med. Assn., 1903, 41, p. 962; Jour. Infect. Dis., 1906, 3, p. 663.

⁴⁷ Lancet, 1913, 1, p. 611.

⁴⁸ Jour. Med. Research, 1905-06, 9, p. 109.

conduct them under identically the same conditions, using a modification of Holman's method with 1% solutions of salicin, mannite, and lactose in carbohydrate-free broth with Andrade's indicator. It will be seen that carbohydrate-free broth was substituted for Hiss serum water, used by Holman. Both methods were tried, but the carbohydrate-free broth was used, since it gave equally as good results and was simpler and cheaper to make.

CLASSIFICATION BY MEANS OF HEMOLYTIC AND FERMENTATIVE CORRELATIONS

Following this procedure and correlating the results, the 51 strains fell into 11 groups shown in table 1 as follows: 20 strains of *S. infrequens*; 5 of *S. pyogenes*; 6 of *S. anginosus*; 6 of *S. subacidus*; 1 of *S. hemolyticus* No. 2; 2 of *S. fecalis*; 4 of *S. mitis*; 1 of *S. non-hemolyticus*; 2 of *S. ignavus*; 3 of *S. equinus*, and 1 of *S. salivarius*. Judging from the number of actual strains in each group, a matter to which Blake called attention, *S. infrequens* could not be considered of minor importance in this series of cultures. Fifty-one strains, however, are probably too small a series for making such general statements.

METHOD OF IMMUNIZATION

The next step was to secure serum for correlating the two previous results with that of a precipitin test. Eight cultures were chosen and rabbits immunized against the cultures, respectively, as follows:

HEMOLYTIC TYPE	NONHEMOLYTIC TYPE
Rabbit 1— <i>S. infrequens</i> culture No. 3	Rabbit 7— <i>S. fecalis</i> culture No. 27
Rabbit 2— <i>S. pyogenes</i> culture No. 2	Rabbit 8— <i>S. mitis</i> culture No. 7
Rabbit 3— <i>S. anginosus</i> culture No. 11	Rabbit 8— <i>S. nonhemolyticus</i> No. 8
Rabbit 4— <i>S. subacidus</i> culture No. 1	
Rabbit 6— <i>S. hemolyticus</i> No. 2 culture No. 26	

The cultures for immunizing were incubated in 20 c c of a 1% glucose veal infusion broth for 24 hours in contrast to Bordet's⁴⁹ method of adding ascitic fluid to broth. The growth was centrifugalized in the same tube as grown, the supernatant fluid discarded, and the sediment re-suspended in 3 c c of salt solution. At first, 1 c c of the suspension was injected subcutaneously over the abdomen every week for 6 weeks. In a few cases the rabbits succumbed to infection,

⁴⁹ Ann. de l'Inst. Pasteur, 1897, 11, p. 177.

notably the first 2 rabbits started on *S. infrequens*. This result contrasts sharply with those of Pasquale,⁵⁰ who observed that short chained streptococci were nonpathogenic. The culture I used in this case was isolated from a gastric ulcer. There were no signs of gastric ulcer in the rabbits after death nor were there any signs of localized lesions except at the point of inoculation; for contrary to results see Rosenow⁵¹ and Thalmann.⁵² These inoculations were followed by weekly intraperitoneal doses for 6 weeks with 2 cc of a 5 cc suspension grown in 20 cc of broth as described, and by subsequent intravenous doses of 2 cc of suspension given daily for 3 days. After an intermission of 3 days the intravenous inoculations were repeated until four such courses were given. Ten days subsequently the rabbits were bled from the heart, about 40 cc of blood being drawn aseptically from each rabbit and allowed to clot. The serum from these bleedings was pipetted off aseptically into sterile containers.

PRECIPITIN TESTS

The serum thus obtained was used in determining the precipitinogen content of the supernatant broth from the 51 strains of streptococci after 48 hours' incubation at 37 C. Two methods were tried, namely, that used by Gay⁵³ in connection with the pneumococcus, and the addition of the diluted antiserum directly to the undiluted supernatant broth from the centrifugalized culture as followed by Blake.⁴⁴ In this work the latter method gave the better result and was therefore chosen. Constant amounts (1 cc) of clear undiluted supernatant broth antigen were added to equal amounts of diluted rabbit antistreptococcus serum. Both homologous and heterologous mixtures were made. Controls consisting of the supernatant broth antigen added to normal rabbit serum, also of normal uncultured broth added to rabbit antistreptococcus serum were made. The following dilutions of serum were made: 1:50; 1:100; 1:200; 1:400; 1:800; 1:1,600, and 1:3,200. The mixtures were placed at 37 C. for from 8-10 hours, then removed to the icebox for 2 hours. In most cases flocculent precipitates were formed in the homologous combinations of the highest dilutions. Heterologous combinations also gave precipitates but few reacted above dilutions of 1:200. Controls showed

⁵⁰ Ziegler's Beit. Z Path. Anat., 1893, 12, 433, p. 499.

⁵¹ N. Y. Med. Jour., 1914, 99, p. 270.

⁵² Centralbl. f. Bakteriöl. I, O., 1912, 46, p. 406.

⁵³ Jour. Exper. Med., 1915, 21, p. 389.

no evidence of precipitate formation. The results (compare table 2) show that specific precipitins were formed, also group precipitins, since in many cases precipitates developed when heterologous combinations of antisera and antigen were made. Sterility tests were carried out to eliminate the possibility of the precipitate being a bacterial growth.

To some of the immune serum 0.3% tricesol was added. This very seriously interfered with the precipitin reaction, so much so that in serum 2, there was not enough to make a complete test with all of the heterologous precipitinogens. In this case the rabbit died after a small bleeding so that no more serum could be obtained.

DISCUSSION

Fifty-one strains of streptococci, isolated from various sources, were examined and classified according to the correlation of their hemolytic, fermentative and precipitinogen properties. Their hemolytic properties were best shown by incubating them for 4 hours at 37 C. in a 5% normal suspension of rabbit corpuscles in salt solution. Two general types were recognized—hemolytic and nonhemolytic. The hemolytic streptococci gave three different varieties of hemolysis. Some of them produced laking of red blood cells without digestion of the stroma, others produced laking followed by methemoglobin formation without stroma digestion, while a third class produced methemoglobin without previous laking, but with stroma digestion. This last type according to Holman⁵⁴ is classified as nonhemolytic when grown on regular blood agar. The nonhemolysers had no apparent effect on the red blood corpuscles. Sugar-free blood agar showed no green pigment producers, but when many of the strains corresponding to the nonhemolysers were grown on the nonfermented blood agar, or on blood containing up to 2% glucose, the green pigment increased in proportion. Hemolytic strains were more frequently associated with acute infectious diseases than the nonhemolytic. The 51 cultures of streptococci were divided into 11 different strains by the correlation of their hemolytic and fermentative test.

The serological investigations consisted in immunizing eight rabbits with one each of 8 of 11 strains and testing the immune sera for their precipitin properties. The rabbits responded by producing anti-serum containing specific precipitins as well as group precipitins.

⁵⁴ J. Med. Res., 1916. 34, p. 377.

TABLE 2
SHOWING HIGHEST ANTISERUM DILUTION WITH POSITIVE PRECIPITIN REACTION

Culture Number of Precipitinogen	Name of Antiserum							
	Infrequens (1)	Pyogenes (2)	Anginosus (3)	Subacidus (4)	Hemolytic No. 2 (5)	Fecalis (6)	Mitis (7)	Nonhemolytic No. 2 (8)
S. infrequens								
3	1:3,200	1:100	1:50	1:100	1:50	1:200	1:200	1:100
4	1:3,200	1:100	1:50	1:100	1:50	1:200	1:200	1:100
5	1:3,200		1:50	1:400	1:50	1:200	1:100	1:100
6	1:3,200	1:100	1:50	1:100	1:50	1:200	1:400	1:200
15	1:1,600		1:100	1:200	1:50	1:200	1:400	1:200
16	1:3,200		1:100	1:200	1:50	1:200	1:200	1:200
21	1:3,200		1:50	1:100	1:100	1:400	1:100	1:100
22		1:100		1:200	1:50	1:400	1:200	1:100
30	1:800	0	1:50	1:1,600	1:50	1:800	1:200	1:400
35	1:3,200		1:50	1:200	1:100	1:400	1:200	1:400
36	1:3,200		1:50	1:200	1:100	1:200	1:100	1:200
37	1:400	1:100		1:200	1:50	1:100	1:100	1:200
38	1:3,200	1:100	1:50	1:200	1:50	1:100	1:100	1:200
41	1:3,200		1:50	1:400	1:50	1:400	1:200	1:200
43		1:800		1:200	1:100	1:200	1:200	1:100
44	1:1,600	1:200	1:50	1:200	1:100	1:200	1:200	1:100
45	1:3,200		1:50	1:400	1:200	1:200	1:400	1:100
46	1:3,200	1:200	1:200	1:200	1:100	1:100	1:200	1:100
50	1:3,200	1:100		1:400	1:800	1:400	1:800	1:200
51	1:3,200			1:400	1:200	1:200	1:400	1:400
S. pyogenes								
2	1:100	1:1,600	1:50	1:400	1:50	1:400	1:400	1:200
10	1:200	1:50	1:50	1:400	1:100	1:100	1:200	1:200
19				1:200	1:50	1:400	1:100	1:100
20	1:200	1:3,200		1:200	1:50	1:100	1:100	1:100
31	1:100	1:200	1:800	1:400	1:50	1:400	1:800	1:200
S. anginosus								
11	1:200		1:1,600	1:400	1:50	1:800	1:400	1:200
29	1:100	1:100	1:3,200	1:200	1:50	1:100	1:200	1:200
47	1:200				1:50	1:100	1:200	1:200
32		1:100	1:100	1:200	1:100	1:200	1:400	1:200
42	1:50			1:200	1:100	1:200	1:200	1:200
49	1:100		1:3,200	1:800	1:50	1:200	1:100	1:200
S. subacidus								
1	1:200	1:200		1:3,200	1:50	1:100	1:200	1:200
18	1:100	1:50	1:50	1:3,200	1:100	1:200	1:800	1:200
23			1:50	—				
25	1:100		1:50	1:3,200	1:100	1:200	1:200	1:400
39	1:200		1:100	1:3,200	1:100	1:200	1:100	1:200
48	1:100		1:100	—	0	1:200	1:3,200	1:100
S. hemolyticus No. 2 26		1:200	1:100	1:200	1:3,200	1:800	1:400	
S. fecalis								
27			1:400	1:400	1:100	1:3,200	1:200	1:200
40	1:200		1:100	1:400	1:100	1:3,200	1:800	1:400
S. mitis								
7	1:200		1:200	1:400	1:100	1:400	1:3,200	1:400
9	1:200		1:50	1:200	1:100	1:400	1:3,200	1:200
17	1:200		1:200	1:400	1:100	1:800	1:3,200	1:200
28	1:100		1:50	1:800	1:100	1:200	1:3,200	1:200
S. salivar 34	1:100	1:100	1:100	1:800	1:100	1:200	1:200	1:200
S. nonhemolyticus No. 2 8	1:200	1:200	1:50	1:800		1:800		1:3,200
S. equinus								
13		1:100		1:1,600	1:100	1:1,600		1:200
14		0			1:100	1:3,200	1:200	1:200
24		1:100	1:100		1:50	1:100	1:200	1:200
S. ignavus								
12	1:200		1:200	1:800	1:50	1:200	1:200	—
33	1:200		—	1:400	1:100	1:400	1:400	1:200

Dilutions of antiserum were only made as high as 1:3,200. Some of the homologous combinations might have been carried still higher, giving higher positive precipitin reactions. Homologous combinations are shown in black face type.

Normal rabbit serum tested for precipitin content gave no reaction. A significant fact is that with few exceptions the specific precipitins closely correlated with the hemolytic and fermentation reactions already mentioned. In general, when immune sera in dilutions up to 1:1,600 and 1:3,200 were added to undiluted homologous antigens flocculent precipitates were brought down, thus showing relatively specific reactions. These reactions outlined in detail in table 2 show the highest dilution of the serum in which a precipitate was observed. The antisera were diluted instead of the antigen (precipitinogen), a reverse from the usual method for testing precipitins.

What the protein complex is in the antigen is uncertain, but we do know that it was less concentrated than the precipitin. This was shown by Gay and Chickering in the concentration of protective bodies in antipneumococcus serum; for after bringing down a maximum precipitate they could still add more antigen and obtain further precipitations. It is interesting to note that the majority of group precipitins failed to react in dilutions above 1:400 and what is of still more interest is the few exceptions as shown by table 2 among the homologous combinations. One thing quite definitely indicated is that streptococci may ultimately be divided into a number of groups with specific metabolic functions and having specific immunizing properties as shown by the results of the hemolytic, fermentative, and precipitin tests now reported.

CONCLUSIONS

Hemolytic tests of streptococci are more readily carried out by incubation in a 5% suspension of corpuscles than on blood agar.

A glucose content of over 1% in blood agar is necessary for the inhibition of hemolysis as compared with sugar-free (fermented) blood agar.

The presence of glucose in blood agar enhances the green pigment production of certain strains of streptococci.

Precipitins produced by immunizing rabbits each with one of several strains of streptococci classified by their hemolytic and fermentation reactions are relatively specific in high dilutions but also give group reactions, in most cases, however, in low dilutions.

Tricresol (0.3%) added to antistreptococcus serum for a preservative deteriorates its precipitins.

The precipitin reaction agrees with the hemolytic and fermentative reactions in classifying streptococci.

DIPHTHERIA AT A HOSPITAL CENTER

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Havard¹ states that diphtheria is "a disease of minor importance in the Army." He quotes statistics to support this view. Keefer² and others, however, find that an epidemic of this disease is at times very difficult to control. It has been the experience of the writers at the Hospital Center, Allerey, Saone et Loire, France, that it requires at times considerable effort, ingenuity and cooperation between the laboratory, ward surgeons, nursing corps and patients, to bring about the desired results. In one-story wooden barracks and tents, covering less than one square mile of space, there were over 17,000 patients and 3,000 personnel at the time that the disease was at its maximum. It is evident that the crowding was very great, and this condition always hampers the control of a contagious disease.

EPIDEMIOLOGY

The first case of diphtheria on record at this Center occurred in Ward V, Base Hospital 26, Sept. 24, 1918. There were two more on Oct. 1, one on the 5th, one on the 8th, three on the 9th, and so on until in the period of Nov. 10 to Dec. 8, from one to seven cases were reported daily. The incidence of clinical cases and carriers at each base hospital is shown in chart 1. Chart 2 is a composite of all the cases in the entire Center. The three hospitals (26, 25 and 49) in which the first cases appeared, were the ones in which more than two-thirds of the entire number of cases developed. It should be borne in mind that since these three hospitals were the first to arrive at this Center, they received the largest number of patients. In these hospitals several nurses and ward men who had been known to be carriers in the States were again identified as such here, but only after the infection had been under way for some time and the culturing had become general. Such chronic carriers might have been factors in introducing the disease at this Center, but it is also possible that they were reinfected here. It may perhaps be unjust that a person once found to be a diphtheria carrier should thereafter always be blamed for any diphtheria developing in the vicinity, but the possibility of course cannot be denied. One of the supposed carriers had only a true Hoffman's bacillus morphologically and culturally, since it did not ferment any of the sugars.

Very early in the epidemic carriers were found with striking frequency among the gassed cases. A number of these cases, diagnosed as diphtheria because of positive throat cultures, came to postmortem examinations and

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¹ Military Hygiene, p. 91.

² Keefer, F. R., Friedberg, S. A., and Aronson, J. D.: Jour. Am. Med. Assn., 1918, 71, p. 1206.

showed either no membrane at all or only a small and clinically insignificant one. Yet from the larynx of these cases practically pure cultures of typical *B. diphtheriae* were obtained. The relative frequency with which such cases were encountered suggested a second possible mode for the introduction of the organism at this Center.

The first ward to be under quarantine for any length of time, due to the constant discovery of new carriers, was the pneumonia ward at base hospital 26, where there were a large number of gassed cases. The same was true in base hospital 56. In base hospital 25 the first cases of diphtheria appeared in the gassed wards. At one time there were 75 known diphtheria carriers (chart 5, B) of whom 33⅓% gave histories of gassing; whereas, of the 35 clinical cases only 5.7% gave such a history. As a possible explanation of the marked preponderance of carriers over clinical cases, it is suggested that the inflammatory changes in the respiratory mucous membrane incident to gassing produces an environment favorable for bacterial growth. The pneumococcus and particularly the streptococcus are invasive organisms frequently causing pneumonia in such cases, but the diphtheria bacillus is relatively non-invasive. Consequently, while the antitoxin producing power of the patient remains unimpaired, diphtheria bacilli may be present in large numbers without causing the disease. Such cases are carriers. It was impossible to go on with this phase of investigation since with the cessation of hostilities no more gassed cases arrived.

Crowding, which means droplet infection, was probably the chief factor in the spread of diphtheria at this Center. During October and early November all wards were greatly overcrowded. Designed for 50 beds, they held 70. To many of the wards were attached one or more tents of 50 beds each, with only just space enough to crowd between every second bed. In the wards, two patients had to share the same table. There were barely sheets enough to cubicle the acute respiratory wards.

When the pneumonia ward of base hospital 26 was first quarantined it was very striking how, over a period of some 2 weeks, carriers continued to appear in two of the tents connected with the ward, whereas the third tent remained uninfected, though cultured as often as the others. This result can be explained only by strict isolation of the uninfected tent from those which were infected. or, due to the crowding, had the organism once been introduced, it would undoubtedly have spread from one end of the tent to the other in a comparatively short time, just as it had in the other two.

The indirect spread of infection by hands and fomites as opposed to direct spread by droplet infection has been known for a long time.³ Fumigation in all its aspects is intended to combat this. But of late the idea that a contagious patient in bed may infect the outmost corners of his room has been replaced by that of direct droplet infection incident to coughing, talking, sneezing, etc., so that only objects in immediate contact with a patient are considered dangerous. Supreme emphasis is therefore placed on the masking of all in attendance. The pendulum has swung a little too far, as our investigation would suggest, from indirect transmission through hands and fomites to direct transmission by means of mouth to mouth droplet infection. This phase of the investigation was suggested by Lieut.-Col. Witherspoon, and it seemed that in the typical diphtheria bacillus we had an unusual opportunity for study, since this organism, we thought, could be recognized in culture by its morphology alone. Also we had here a considerable number of clinical cases and contacts under far better control than is possible in civil life.

³ Park, W. H., and Williams, A. W.: *Pathogenic Micro-organisms*, 1917, p. 297.

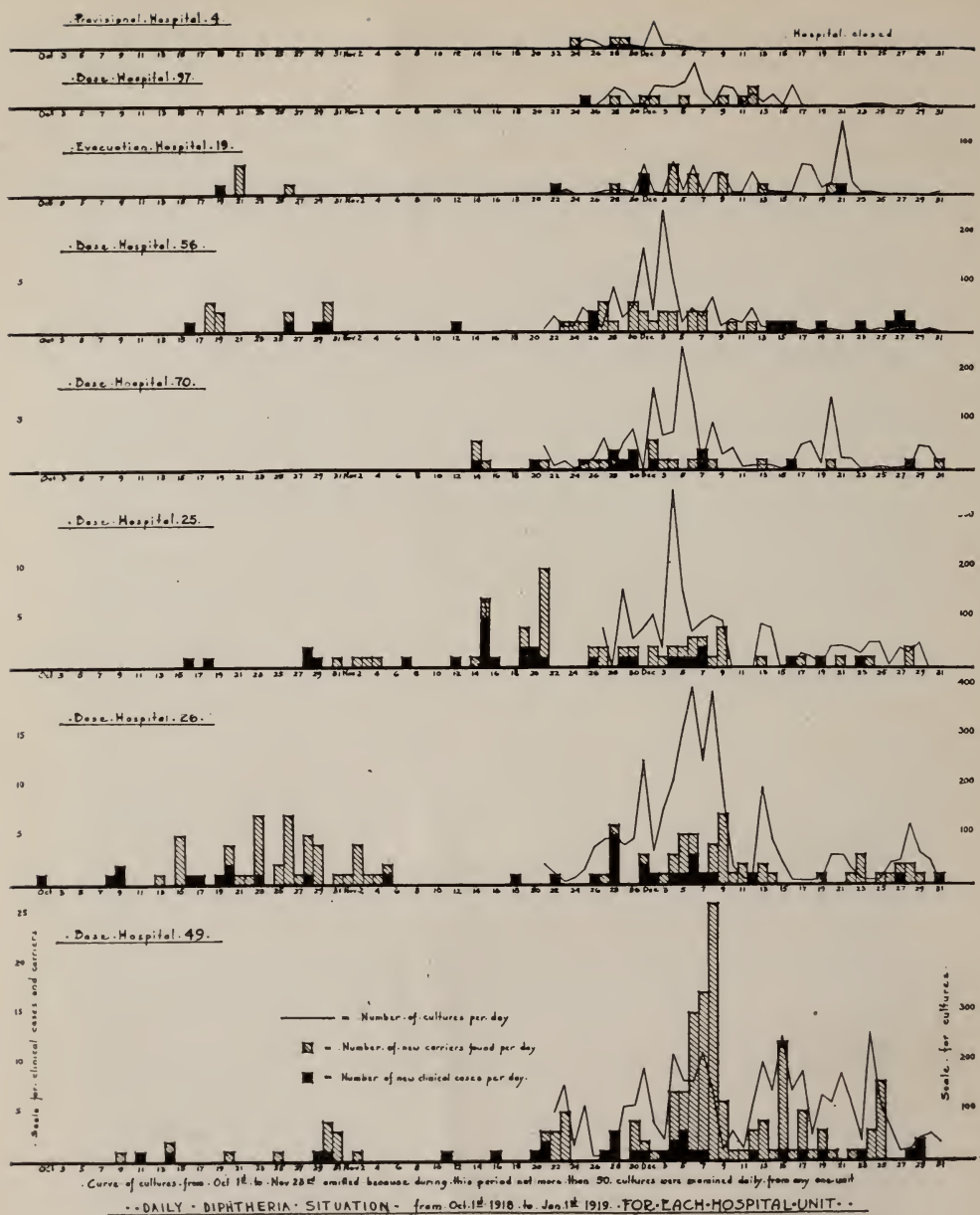


Chart 1.—It should be noted that base hospitals 49, 25 and 26 furnished most of the clinical cases and carriers. Up to Nov. 6, three to four carriers were found at base hospitals 49 and 26 for each clinical case, whereas, compared with the number of clinical cases, base hospital 26 showed few carriers. This may in part be accounted for by the less general culturing at this hospital at this time. The clinical case at base hospital 26 recorded on Oct. 1 was the first case at this Center and should have been recorded on Sept. 24, had the chart permitted. The first carrier at provisional hospital 4 was sent to base hospital 56 as a clinical case, but it was decided to be merely a follicular tonsillitis with a positive culture. On Dec. 6, 7 and 8 the marked increase in carriers at base hospital 49 was due to the great prevalence of the atypical forms. The same were found Dec. 8 at base hospital 26, when the carelessness was shown in taking cultures. These were never reported positive, since the ward was quarantined and all throats cultured. The same procedure was adopted at base hospital 49, as is shown by the marked drop in the curve of cultures Dec. 9. The most recent cases recorded at base hospital 56 were in patients held in the cubicle and mask observation ward, which may account for the absence of new carriers. It should be noted that in all instances the sum of chart 1 will not equal chart 2. This is because some 13 of the first clinical cases could not be traced back to the base hospital where they originated, and some few cases were sent in from the convalescent camp, which is not shown on chart 1.

The diphtheria wards, both clinical and carrier, were chosen for this investigation, using wards in which no cases of diphtheria had developed as controls. In all, 522 cultures were made. The following procedure was adopted: a sterile swab moistened in sterile broth was wiped over the palm, particular attention being paid to the creases. It was felt that owing to heat and moisture the creases would be most likely to retain the organisms which might be deposited there. The swab was inoculated on Loeffler's blood serum and incubated from 16-18 hours. Working on the principle suggested by Williams,⁴ some 90 cultures were enriched after 8 hours' incubation by inoculating fresh tubes with a loop passed over the surface of the original one. Both series of tubes were examined from 10-12 hours later. The enriched series did not show a constant increase in the number of positives and the procedure was discontinued. A few cultures were first taken from the fingers also, but this was soon abandoned because no additional information was obtained. It was felt that contamination of the fingers could be reasonably assumed once the palm was proved positive.

All the attendants and nearly all the patients were masked in the clinical and carrier diphtheria wards. A few of the bed patients who were cubicled were not masked. Occasionally the patients were asked to cough before the cultures were taken. Patients without masks and often patients with masks would unconsciously place their hands on their mouths when coughing. At times they were explicitly told to cough into their hands. This was done routinely toward the latter part of our investigation.

For purposes of comparison patients were divided into two groups: Those in "infected" wards, and those in "uninfected" wards. We use the term "infected" wards to mean wards in which clinical cases or carriers were isolated. Base hospital 56 furnished the "infected" wards because all clinical and carrier cases from the Center were sent there. The term "uninfected" refers to wards in which no clinical case or carrier had been identified at any time; such wards were selected from hospitals in which there had been the least amount of diphtheria. These groups were again divided into "masked" and "unmasked." Unmasked patients were rather hard to find in the diphtheria wards, with the exception of the cubicled bed patients. In the "uninfected" wards chosen, all were unmasked. Fomites were similarly subdivided into "masked" and "unmasked." Only articles used or handled by masked patients were considered "masked" fomites. Such articles included door-knobs, handles on latrine pail covers, pokers, crutches, and personal possessions such as mess-kits, mirrors, rosaries, magazines, foot-rails of beds, etc. The personal possessions of unmasked bed patients were considered "unmasked" fomites. This distinction is entirely arbitrary, since cigaret papers, spoons, mirrors, combs, etc., are used when the mask is off and the patient who is masked at the time of culture may have had his mask off or hanging from one ear only 5 minutes previously.

The typical diphtheria bacillus when present in these cultures was found only after considerable search. They were usually in clumps of three or more, showing polar-staining and parallel or stellate arrangement. The majority were of the smaller forms corresponding to Westbrook's "C" or "D". The atypical diphtheria bacillus or diphtheroid was similar in morphology to that found in the throat, in the mouth, in the anterior nares, and in several of the wounds. It resembles the diphtheroid described⁵ as the normal inhabitant of the skin.

⁴ Ibid., p. 306.

⁵ Eberson: *Jour. Infect. Dis.*, 1918, 23, p. 1.

The number of the organisms showing metachromatic polar granules was very surprising. Some of these were often indistinguishable from the large polar-staining diphtheria, though the frequency of a large metachromatic granule at the center made one at once suspicious. Also the majority did not show the characteristic tapering toward the center nor the usual slight curve. When the grouping of these organisms in the smears was considered, the absence of all palisade arrangement was striking, the bacilli often appearing in chains of two, three or even more. Spore-bearing bacilli were found

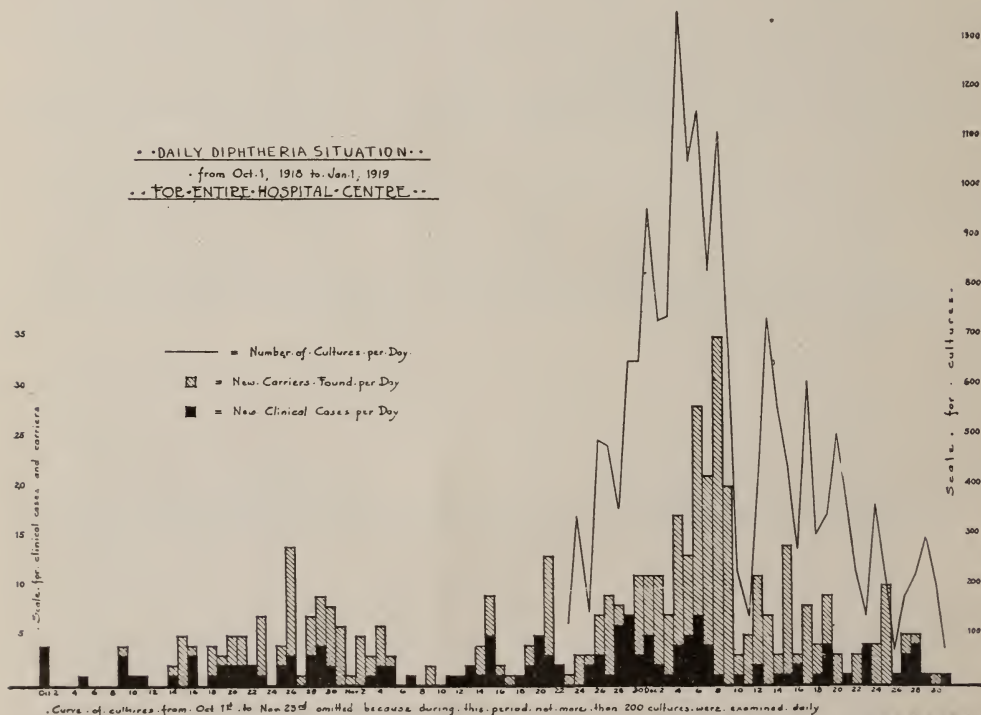


Chart 2.—Note that in general the curve of new carriers found follows that of the number of cultures examined. The marked exception is on Dec. 8, when the atypical form was being found in great numbers at base hospital 49. In Oct. and early Nov. the culturing was much less general, only those immediately exposed being cultured. Hence, there was at that time a much higher proportion of positives.

in the same smears. These two forms were occasionally the only organisms present. It is, of course, unreasonable to expect to recover an almost pure culture of diphtheria bacilli in cultures from the foot of a bed or from the hand of a patient, even when the patient is known to harbor this organism in his throat. It was felt that the spore-bearing and the polar-staining bacilli were probably only different forms of the same organism—a saprophyte corresponding to Bizzozero's *Leptothrix epidermis* and not a diphtheroid at all. To prove this, isolation was attempted. Isolation through plating failed because of the extremely spreading character of the growth. Isolation of the spore-bearer through heating, however, was successful. Suspensions of the

culture in salt solution were heated for half an hour at 70 C. and then planted out on Loeffler's blood serum. As controls were used cultures of typical diphtheria from a clinical case which killed a guinea-pig in 36 hours; a culture of typical diphtheria recovered from a larynx at necropsy; a culture from a wound showing type "D2" organisms. All the controls showed no growth in the subcultures at the end of 72 hours. The subculture of the spore-bearer, however, showed an abundant growth at the end of 24 hours with both the polar-staining and the spore-bearing forms present. These were identical with the original culture. Cultures from the foot of the bed, from a hand, and from a rosary, all showing these two forms, had the same forms in the subcultures after heating. This gave us convincing proof that this polar-staining form was not of the diphtheria group at all, though in occasional instances it would have been extremely difficult to exclude it without the examination following heating.

Chart 3 shows results of the 522 cultures from hands and fomites; 16.1% of the "unmasked" hands in "infected" wards showed typical diphtheria, while only 6.4% of the "masked" hands showed it, thus indicating the value of masking in preventing droplet infection of the hands. Of the cultures from unexposed wards, 5.4% showed a diphtheroid and none typical diphtheria bacilli. The incidence of this diphtheroid in cultures from exposed wards was much higher, 10.3 %; in these wards patients were known to harbor a morphologically similar organism. However, since these organisms or similar organisms are normal inhabitants of the skin, the results of the investigation as far as this organism is concerned, may be somewhat misleading, because its presence may be accounted for otherwise than by droplet infection from the throat. But when a typical diphtheria bacillus is found on the hand and at the same time is known to be an inhabitant of the patient's throat, it is reasonable to assume a direct transmission from throat to hand by coughing or sneezing, and thence to fomites by the contaminated hand. As noted above, this study would emphasize the value of masks in preventing droplet infection of hands and probably of fomites, but whether direct droplet infection from mouth to mouth or indirect droplet infection by way of the hands and fomites is the principal mode of spread must still be little more than a matter of individual opinion. However, this does emphasize what has long been known—the importance of the indirect method of spread of diphtheria. Greater attention must be paid to this manner of disseminating contagious diseases.

A third factor in spread, once the organism had been introduced, was late diagnosis. Because of the considerable number of gassed patients in the hospitals, recognition of laryngeal diphtheria was at times extremely difficult. This was due to the great similarity between the two diseases, not only in the mechanical obstructive symptoms which developed but also in the appearance of the membranous exudates. The severely gassed cases often developed fibrino-purulent membranes which covered the lining of the larynx and trachea, extending from the epiglottis down into the bronchi and bronchioles. The aryepiglottic folds and tissues about the ventricles and vocal cords were at times markedly edematous, producing voice changes, hoarseness and mechanical obstruction to breathing. This membranous exudate rarely extended upward into the pharynx and never over the tonsils. But nearly all of the serious cases of diphtheria had severe laryngeal involvement, often without any pharyngeal manifestations, so that even at necropsies it necessitated close scrutiny to differentiate between the two types of lesions. Besides, diphtheria was sometimes superimposed on the laryngitis following gassing. It is there-

Cultures from Hands and Fomites.

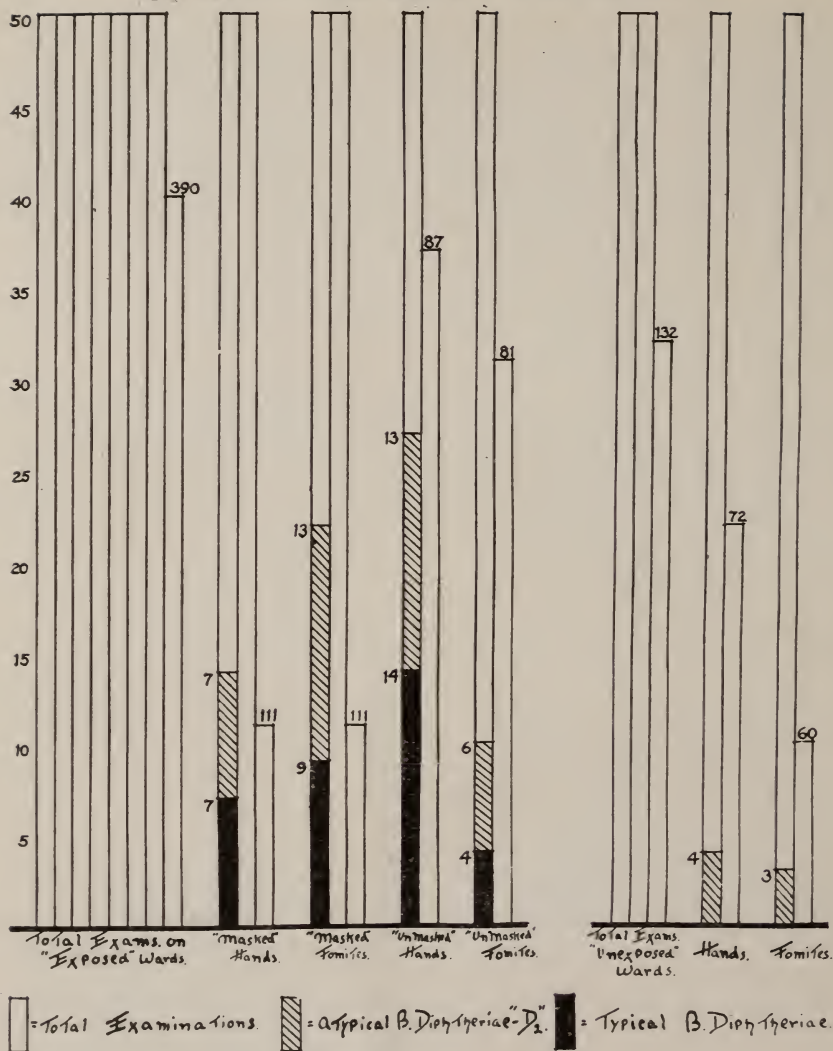


Chart 3.—"Exposed" Wards: Typical B. diphtheriae on "masked" hands, 6.3%; on "masked" fomites, 8.1%; Typical B. diphtheriae on "unmasked" hands, 16.1%; on "unmasked" fomites, 4.9%; Atypical B. diphtheriae on "masked" hands, 6.3%; on "masked" fomites, 11.7%; Atypical B. diphtheriae on "unmasked" hands, 14.9%; on "unmasked" fomites, 7.4%. "Unexposed" Wards: Atypical B. diphtheriae on "unmasked" hands, 5.9%; on "unmasked" fomites, 5.0%. "Exposed" wards were those in which the clinical cases of diphtheria or carriers were treated. "Unexposed" wards were those in which no cases of diphtheria or carriers had been found at any time. The term "masked" means that the patient whose hands or fomites were cultured wore a mask, while "unmasked" means conversely that he wore no mask. It should be noted that typical B. diphtheriae was found nearly three times as often on the hands of those not wearing masks as on those wearing them. Nearly the same difference was found with the atypical form. With the fomites the "masked" were more often infected. This may be explained in part by the fact that many of the fomites considered "masked" were used only when the patients were unmasked. None of the control cultures on "unexposed" wards showed typical B. diphtheriae.

fore easily seen that there were great difficulties in differential diagnosis. As a result an order was sent out that on admission to the hospital all gassed cases must be cultured for diphtheria.

Three cases came to necropsy in which laryngeal diphtheria was found to be the cause of death but which were not diagnosed clinically. Two more patients died of laryngeal diphtheria in which the diagnosis was made only 24 hours before death. Such cases obviously are a great menace in congested wards. Another case of delayed diagnosis was that of an enlisted man who came to the laboratory for examination accompanied by an attendant from one of the medical huts. He had been sick for 3 days, sitting around the stove in his barracks. There was marked respiratory stridor, some aphonia, and considerable swelling of the neck which had been painted with iodine. On inspection he showed a perfectly characteristic membrane over both tonsils extending up onto the soft palate. Direct smears from the exudate showed the diphtheria bacillus. Such experiences of delayed diagnoses help explain the spread of diphtheria in spite of all other precautions.

BACTERIOLOGY

During our study of diphtheria at this Center, beside the typical polar-staining bacillus of Klebs and Loeffler, we found a small diphtheroid in large numbers from cultures of the nose and throat. Our attention was called to this organism early, when a ward at base hospital 49 was cultured. Two cases showed this bacillus in practically pure culture with no typical polar staining, and the ward was pronounced negative. Three days later a new clinical case appeared in this ward. This led us to report the first culture, in which this diphtheroid appeared as "Diagnosis reserved," and to request a new culture. If the organism persisted, the report "Positive atypical" was made. This arbitrary standard was to be followed until we had time to obtain results as to its virulence and its frequency in clinical cases.

This diphtheroid is a lancet- or wedge-shaped diplobacillus slightly resembling pneumococcus but somewhat longer in proportion to its width. It shows the palisade arrangement characteristic of the diphtheria group, and tincorially and morphologically corresponds to "D₂" in Westbrook's classification. It is gram-positive, nonmotile, shows no spores and no capsule. As a routine in the laboratory we used Barron's polychrome toluidin-blue⁶ diluted 1:5, which stains it uniformly except for the midfissure. There are neither barred nor polar forms. We have found that this form may go over into the typical staining organism, may be mixed with it, or may remain entirely true to type. For example, a throat culture has not infrequently been brought to the laboratory in the morning, which after some 8 hours' incubation showed only the wedge forms; whereas, on the following morning only typical polar forms were present. Again, at base hospital 26 one of the gassed cases, followed by pneumonia, was negative when examined in a routine culture of the quarantined ward. Two days later the patient showed a few of the atypical forms, and these were practically in pure culture the next day. A culture on the

⁶ Jour. Lab. and Clin. Med., 1918, 3, p. 432.

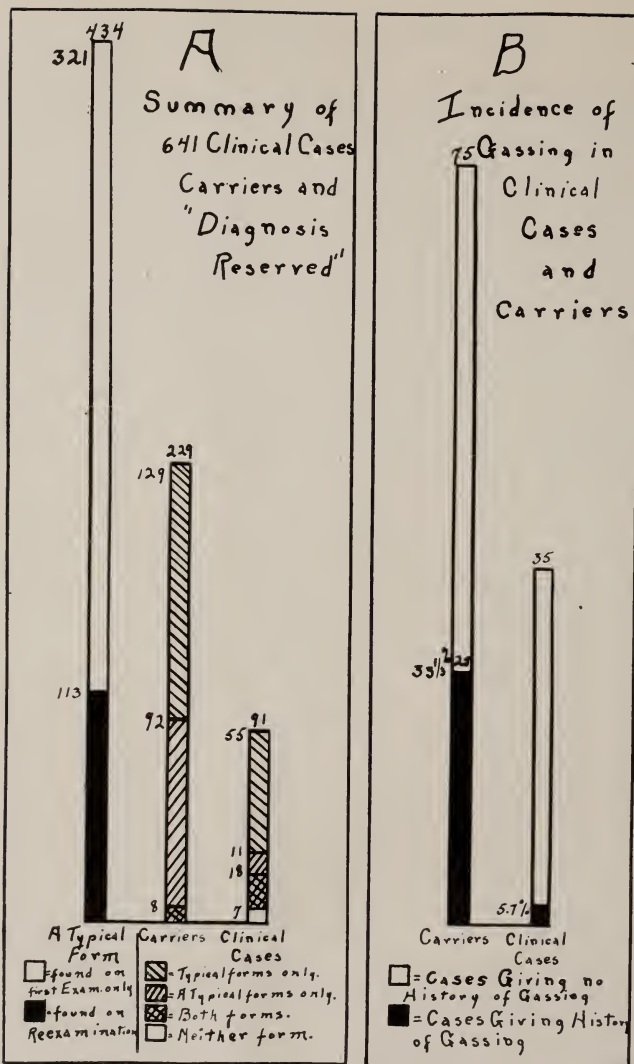


Chart 4.—A: "Diagnosis reserved" was the report given on cultures when the atypical form was first found. A second culture was requested and if the organism persisted the 2nd day the report "Positive atypical" was given. The first column shows that in about three out of every four cases the atypical form did not persist, that is, it was but a transient inhabitant of the throat. Note that the atypical form only was found in 56.1% of the carriers and 60.4% of the clinical cases; the atypical form only appeared in 40.1% of the carriers, and 12% of the clinical cases; both forms were found at different times in 3.8% of the carriers and 19.7% of the clinical cases. The 7.9% of the clinical cases in which no organism of the diphtheria group was found is accounted for as follows: At first all the individual cultures, among which were most of the clinical cases, were examined at the unit laboratories. If found positive the patient was sent to the contagious hospital, where, owing to the stress of work, no other culture often was taken until the patient was convalescent and ready for discharge. In such cases he would not appear on our records until late and then occasionally as having only negative cultures. B: This chart is made from patients found in the carrier and clinical wards at a given time, and not from selected cases. Unfortunately the percentage of gassed cases of the total number of patients in the hospital was unobtainable. But it is striking that although the carriers and clinical cases came from the whole Center at large, 33 1/3% of the carriers gave a history of gassing, while only 5% of the clinical cases gave such a history.

following day showed typical *B. diphtheriae*. At necropsy smears from the larynx showed the characteristic organisms, though there was no evidence of any membrane. Some of the throat cultures showing only wedge forms in original culture showed a mixture of wedge, polar, barred and club forms after isolation in pure culture (chart 4).

The cultural characteristics of this form are not unlike those of typical diphtheria. On Loeffler's blood serum, at the end of from 12-16 hours, it shows small, discrete, glistening, buff-gray colonies, slightly raised. Toward the butt of the tube, where the medium is moist, they become confluent. On broth there is moderate clouding, at times flocculation, but no pellicle formation. The relation of this organism to the typical diphtheria bacillus is one of great interest. Table 1 shows the morphology and sugar reactions on 14 cultures isolated early. Six are from clinical cases, of which, in the original cultures, 4 showed the typical organisms, and 2 the wedge form only. When isolated in pure culture all showed the typical form. The other 8 cultures were from carriers, 2 of which showed typical diphtheria, and the 6 others showed a preponderance of the wedge form.

Technic of Isolation.—A small loop of material from the original throat culture was streaked out on one-half of a 1% dextrose-agar plate, to which one-half of 1 cc of human serum had been added. From this a suspicious colony, usually pinhead size or smaller, discrete, translucent, of a bluish, skim-milk color, was streaked on half of a second plate. From this a colony was streaked on Loeffler's blood serum and after from 16-18 hours' incubation was examined for morphology.

Fermentation Reactions.—Pure cultures were inoculated into tubes of 10 cc of Hiss' serum water to which $\frac{1}{2}$ cc of a 20% sugar solution, sterilized fractionally, had been added. One-tenth of 1 cc of sterile Andrade indicator was added on the 2nd day. The tubes were read after 2, 4, 6 and 8 days of incubation. On the 8th day smears were examined for contamination.

It has been the experience of one of us in a study of diphtherial infection in wounds that the saccharose fermentation was of considerable importance as regards virulence, that is, that nonsaccharose fermenters are virulent and most saccharose fermenters are avirulent or only slightly virulent. Because of the difficulty in obtaining guinea-pigs and the unsatisfactory results obtained in intracutaneous virulence tests, only five cultures were tested. As chart 4 shows, two of the virulent strains, killing in 24 and 36 hours, respectively, were nonsaccharose fermenters, one being morphologically typical diphtheria, the other showing only wedge forms. The third culture which showed only wedge forms in original throat culture killed in $4\frac{1}{2}$ days, and fermented saccharose. The other two strains were pure cultures of the atypical organism and had not killed at the end of 2 weeks. All control pigs were negative.

Technic of Virulence Tests.—Forty-eight hour infusion broth cultures were used. One-half of 1% of the body weight of each pig reckoned in cc was injected subcutaneously. A proportional amount of each culture was injected subcutaneously into a control pig which had been immunized 24 hours previously by an intraperitoneal injection of 100 units of antitoxin. From the pigs that died, cultures were made from the subcutaneous tissues at the site of injection, from the peritoneal fluid, and from the heart blood. The organism injected was recovered only from the one dying after $4\frac{1}{2}$ days. The adrenals were congested in all three cases.

TABLE 1

	Morphology		Fermentation Reactions																Virulence for Guinea-Pigs		
			Saccha-rose	Dex-trin	Dex-trose	Lac-tose	Mal-tose														
	Days	Days						Days	Days	Days											
											2	4	6	8	2	4	6	8		2	4
B Clinical	Practically pure culture D ²	A ₁ B ₁ C ₁ Rare B & C	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	+	+	+	+
E Clinical	B C	B C A ₁ B ₁ C ₁	—	—	—	—	±	+	+	+	+	+	+	+	+	+	+	—	+	+	+
S Clinical	D ₂ Rare C ₁ D ₂	A ₁ B ₁ C ₁ Rare B C D	+	+	+	+	—	—	—	—	+	+	+	+	+	+	+	—	+	+	+
S ₁ Culture from guinea-pig		B C B ₁ B ₂ C ₁ C ₂	—	+	+	+	—	—	—	—	+	+	+	—	+	+					
T Clinical	B ₁ C ₁ Rare B C	B C A ₁	—	—	—	—	—	+	+	+	—	+	+	+	—	+	+	+	—	+	±
G Clinical	B C	B C B ₁ C ₁	—	—	—	—	—	+	+	—	±	+	+	+	—	—	—	±	+	+	+
W Clinical	B C	B C B ₁ C ₁	—	—	—	—	—	+	+	+	—	+	+	+	—	+	+	±	+	+	+
W. Y. Carrier	D ₂	C ₁ D ₁ C ₂ D ₂	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+
W. R. Carrier	D ₂	D ₂	—	—	—	—	—	+	+	+	—	+	+	+	—	+	+	—	±	—	—
A Carrier	D ₂	D ₂	—	+	+	+	—	—	—	—	—	+	+	+	+	+	+	—	—	±	+
K Carrier	D ₂	D ₂ Rare C ₁ D ₁	+	+	+	±	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+
C Carrier	D ₂	D ₂ Rare C ₂																			
P Carrier	D ₁ D ₂ Rare D	C ₂ D ₂																			
E. K. Carrier	B C	A B C A ₁ B ₁ C ₁	—	—	—	—	—	+	+	+	—	+	+	+	—	+	+	±	+	+	+
W. A. Carrier	B C	B C	+	+	+	—	—	+	+	+	+	+	+	+	—	—	—				

Table 1.—The fermentation reactions are different from those commonly given, in that B. and W. A. ferment both saccharose and dextrin, and ten of the cultures ferment lactose. The two most virulent strains, E. and W. Y. do not ferment saccharose; the less virulent, S., and avirulent, K. and W. A., do not ferment saccharose. Cultures C. and P. are morphologically and culturally B. Hoffmanni. Note that the virulent cultures S. and W. Y. show the wedge form (D-2) predominatingly or in pure cultures in the original throat cultures, while K. was avirulent. Cultures E. and W. A., both typically B. diphtheriae, were virulent and avirulent respectively.

The other data bearing on the virulence of this atypical organism were for the most part clinical. Chart 4 A gives a summary of our laboratory records up to Dec. 10. Of 434 cases in which the atypical organism was found, only 113 showed it on the following day, that is, in 75% of the cases it was only a transient inhabitant of the throat. Of the eighteen clinical cases showing both typical and atypical organisms, ten, or 11.1%, of all the clinical cases showed the atypical organism first and later the typical. In eight cases the reverse was true. It cannot be denied that in the ten cases the appearance of the typical form later can be explained by a fresh infection with this organism, since these cases were in the clinical diphtheria ward where cross infection was possible. But in these ten cases the atypical form was able to produce symptoms sufficiently suggestive of diphtheria to warrant their being admitted to the clinical rather than to the carrier ward, and we have had frequent evidence that at times this wedge form is one of the many forms that may be assumed by the extremely pleomorphic typical diphtheria bacillus.

On Dec. 6, 7 and 8 (chart 1), base hospital 49 showed a sudden increase in the number of atypical forms—one ward having 30. At the same time base hospital 26 showed a similar increase. On inquiry at the latter hospital it was found that the "team" taking the cultures had become somewhat discouraged at the large amount of work without apparent results, and had decided that it was time for the ward to come out of quarantine. With this end in view they gave up the use of tongue depressors and cultured at random about the tongue and teeth and in the anterior nares. The result was that all three wards cultured on that day showed from 15-25 cases of "Diagnosis reserved." One of these wards, cultured properly, had been entirely negative the day before. No such explanation could be found, however, in the case of base hospital 49. Morphologically this organism found in the mouth and anterior nares could not be distinguished from the wedge form we had been finding in the throat. Also the diphtheroid found on hands and fomites in wards where there had been no diphtheria, had the same morphology.

It is our opinion that cases showing this atypical organism in properly taken nose and throat cultures must be treated as positive diphtheria cases or carriers until each strain has been proved nonvirulent, since (1) two pure cultures of this organism killed guinea-pigs in 24 and 108 hours, respectively; (2) 11.1% of the clinical cases showed this form only at first and later showed the typical organism, and (3) variation in length of incubation may show first the atypical and then the typical forms. However, in the majority of cases, this form was only a transient inhabitant of the throat and, we believe, was avirulent.

METHODS OF CONTROL

From the first, base hospital 56 was chosen as the contagious hospital for the Center and both clinical cases and carriers were sent there. The wards in which these were discovered were quarantined and cultured as directed by the head of the medical service in each hospital. It was early seen that much would be gained both in uniformity of results and in the availability of statistics by having the cultures examined at the central laboratory for the Center rather than by the several unit laboratories. Up to the first of Dec. all the cultures were examined by one man. Since then, owing to the increased

number, it was found necessary to have two examiners who constantly consulted with each other on doubtful cases.

Toward the latter part of Nov., when from 1-7 new cases developed each day, it was felt that the methods of control at the various hospital units should be more rigorous and more uniform. It was decided to include as clinical cases all those with positive cultures and throat symptoms, though such symptoms might be very slight. Three methods of control were emphasized, any one of which by itself should, theoretically, control spread of infection.

1. *Individual Quarantine*.—The face^{7, 8} mask epitomized individual quarantine. The quarantine of the ward from communication with the outside seemed to be fairly well understood and carried out, but the quarantine of the individuals within the ward from each other was almost completely neglected at that time. For instance, on the discovery of a clinical case the ward was isolated and cultured. Usually one or more positive carriers were discovered and reported in 24 hours. But during this 24 hours the patients who were up would congregate around the stove, owing to inclement weather, playing cards, waiting on the bed patients, and so on. The carriers who were about to be detected had been infecting others by droplet and hand. In other words, at the time when the ward culture was received at the ward it gave no accurate idea as to the number of individuals carrying the diphtheria bacilli in their throats. The same was true of the report on the second ward culture, and so on, ad infinitum. One ward was cultured four times in 7 days. The first culture showed 1 carrier; the second, 2; the third, 2, and the fourth, 4. After each report the carriers were evacuated to the contagious hospital, and it seemed to be only a question of time and endurance before the entire ward would be transferred.

The mask^{9, 10} was suggested as a remedy. It was, of course, impracticable to mask all of the 16,000 individuals at the Center, though one hospital tried masking its entire personnel. The dissociation of mask from quarantine was found detrimental to the discipline and mental attitude, which is not the least important function of the mask. This might be termed "mask psychology." Cubicles were recommended to supplement the mask, since few can sleep with the

⁷ Capps, J. A.: Jour. Am. Med. Assn., 1918, 70, p. 910.

⁸ Weaver, G. H.: Ibid., 1918, 70, p. 76.

⁹ Doust, B. C., and Lyon, A. H.: Jour. Am. Med. Assn., 1918, 71, p. 1216.

¹⁰ Haller, D. A., and Colwell, R. C.: Ibid., 1918, 71, p. 1213.

mask in place. It was urged that the patients be kept by their beds as much as possible, particularly during meals, when, of course, the mask must be removed. The original masks had two layers of gauze with a mesh 14 by 11 threads per cm. It was recommended that two such masks be worn, and that all future masks be made with four layers. Our first point, then, was individual quarantine as represented by mask and cubicle.

2. *The Schick Reaction.*—Theoretically, if we had examined every individual at the Center by the Schick test and immunized all positives with a prophylactic dose of antitoxin, the spread of diphtheria would have stopped at once. The detection of carriers would have been of no interest, since there would have been no one for them to infect. But this was not considered, because for some time it was questionable whether enough toxin was available for the quarantined wards alone, as had been recommended. A control of inactivated toxin was quite out of the question. The value of the Schick reaction without such a control was the subject of considerable discussion. It was felt that since our object was to stop the spread of the disease at this Center and not to obtain data as to the value of the Schick test, that by reading the reaction at 24, 48 and 72 hour intervals we could divide the reactions into three classes: Those frankly positive; those frankly pseudopositive, and those that were questionable. By immunizing cases showing a positive or a questionable reaction, we were erring on the side of safety. On 1,363 Schick tests reported from five of the hospitals, the percentages of positives were as follows: 8% at three hospitals, 6% at one, and 0.6% at another. Obviously, there was a flaw in the technic of the test at the last named hospital. There were a few carriers showing positive Schick tests, but shortage of guinea-pigs prevented our testing their cultures for virulence. Also an occasional patient having a negative Schick test came down with diphtheria a few weeks later. The explanations for this are either faulty technic, inert toxin, or overwhelming infection. Our second method of attack, then, was the Schick reaction plus prophylactic immunization.

3. *Nose and Throat Cultures.*—One of the earliest suggestions for the control of diphtheria was that all of the 16,000 individuals be cultured. Because of our limited autoclave space our daily output of medium could not be pushed beyond 2,000 tubes. There was also a shortage in personnel, both officers and enlisted men. It would, there-

fore, have taken 8 days at the very least, to culture the entire Center. Thus we would have had the same difficulties which we experienced in the wards before individual quarantine was instituted, only multiplied 8 times, since it was out of the question that during this entire period each individual at the Center would refrain from all except "masked" communication with all the others. At the end of 8 days, our summary report on the names and number of carriers at the Center would have no value, since during the 24-hour interval between culture and reports, each "positive" would have infected an unknown number of other individuals. So this plan was fortunately abandoned. Definite rules as to the number of times a ward was to be cultured following each new case were formulated. Emphasis was laid on a short, rigorous quarantine since it was felt that the effectiveness of a quarantine is in inverse ratio to its length. Personal cooperation of the patients is absolutely essential to individual quarantine, and this would be entirely lost unless they understood what is being done, why it is being done, and that the hope of release is reasonably within sight.

These rules were helpful not only in standardizing quarantine, but also in stemming the flood of cultures. There is always the danger that wholesale, promiscuous and nonselective culturing may give a false sense of security. For a period of 10 days—from Nov. 29 to Dec. 9—600 to 1,300 cultures were examined daily. The positives each day varied from 1.1-3.1% of the total cultures examined. The last 4 days of this period, when the percentage rose as high as 3.1% a large number of atypical forms was being found, partly accounted for by the careless mouth and anterior nares cultures previously mentioned. During the first six days the percentage varied only from 1.1-1.8%. If from this be subtracted the 0.3-0.6% of clinical cases each day, there is left a daily quota of carriers amounting roughly to 1% of those examined. This is what Rosenau¹¹ gives as the normal percentage of diphtheria carriers among the unexposed population. In other words, what we really had here was not a potential epidemic of diphtheria but an actual epidemic of culturing. Our third point in control is selective nose and throat cultures, properly taken.

Finally, the most important measure in the suppression of any epidemic is early diagnosis. This is of special significance in a disease such as diphtheria, in which the spread by contact is unquestionable, and, in addition, early diagnosis spells practically 100% cures.

¹¹ Preventive Medicine and Hygiene, 1918, p. 1374.

The influence of delayed diagnosis on spread has already been discussed. To emphasize this, a special order was issued that the throats of all patients and personnel be inspected daily. All suspicious cases were to be isolated and cultured. Observation wards were established where such patients were held pending the report on their cultures. Such wards were strictly cubicled and masked, thus reducing contact infection to a minimum.

CONCLUSIONS

The two probable modes of introduction of the diphtheria bacillus into this Center were "gassed" carriers and chronic carriers.

Factors in spread were crowding (direct droplet infection) contaminated hands and fomites (indirect droplet infection), and delayed diagnosis, partly explained by the similarity in symptoms between laryngeal diphtheria and membranous laryngitis due to gassing. Of these we consider crowding the most important.

Morphology of the diphtheria group is no criterion of virulence. Occasionally, difficulty was encountered in distinguishing between polar-staining, nondiphtheria bacilli and typical *B. diphtheriae* on morphology alone. Such confusion was experienced only in cultures from hands and fomites, but never in throat cultures.

The four methods of control employed were:

1. Early diagnosis through frequent throat examinations.
2. Individual quarantine exemplified by the mask and cubicle.
3. The Schick test followed by immunization of susceptible individuals with antitoxin.
4. Selective culturing of nose and throat carefully performed.

INFLUENZA IN THREE CHICAGO GROUPS *

EDWIN O. JORDAN, DUDLEY B. REED AND E. B. FINK

Opportunity has been afforded us for the study of influenza prevalence in three different population groups in the city of Chicago: (1) The Student Army Training Corps at the University of Chicago; (2) The High and Elementary Schools of the University of Chicago, and (3) the Chicago Telephone Company. These several groups are quite distinct as regards age, degree and nature of association of the individuals within each group, and general opportunities for exposure to infection. So far as known there was no point of contact between the groups.

I. THE STUDENT ARMY TRAINING CORPS

EDWIN O. JORDAN AND DUDLEY B. REED

In October, 1918, influenza broke out in a group of the Student Army Training Corps at the University of Chicago. This group, known as Section B, comprised 234 men, nearly all about 20-22 years of age, coming mostly from small cities, towns and rural districts in Illinois. They were housed in 5 different places—remodeled houses and apartment buildings. The number of occupants to a room varied somewhat, being usually about 4-8. Although sleeping in five separate buildings, they were all closely associated in their technical class work and at meals. The men for the most part came directly from their homes, arriving in Chicago October 15 and 16. Three of the men were ill on their arrival, in two cases with symptoms that as described seem influenza-like. On the evenings of October 16 and 17 all men in the group were brought into especially close contact in the locker room of the University gymnasium while waiting for their physical examination.

The date of onset was determined by individual questioning in each case and could usually be fixed by the patient within a few hours as is characteristic of influenza. The cases developed as follows:

Date of Onset	No. of Cases	Date of Onset	No. of Cases
Oct. 15 }	3 (Ill on arrival in Chicago)	Oct. 20	22
Oct. 16 }		Oct. 21	13
Oct. 17	10	Oct. 22	4
Oct. 18	14	Nov. 4.....	1
Oct. 19	29	No definite information.....	9

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* Influenza Investigations, U. S. Public Health Service.

The situation became known to the University authorities on October 20, and all the affected men were removed to an emergency hospital.

Daily temperatures were taken of the rest of the group for one week. Isolation followed any sign of fever. The number of men in each building at the beginning of the outbreak and the corresponding number of cases is as follows:

Building	No. of Men	No. of Cases
A	15	5
B { Upper	61	29
{ Lower	30	11
C	19	4
E	40	17
F	51	16

In each of three buildings (Lower B, E and F—121 men) one man was ill on arrival; in these houses 8 cases developed on October 17; in the remainder (A, Upper B and C—95 men) with no known cases of illness before October 17, two cases developed on the 17th. In the later cases the time and place distribution did not give any indication that infection occurred principally in sleeping quarters.

The chief symptoms accompanying onset were (100 cases):

Headache	66
Muscle Pains.....	56
Sore Throat.....	37
Cough	34
Nosebleed	8

Fever ranging from 100-104 F. was present in all these cases. The face was usually deeply flushed and the conjunctiva more or less injected.

Information was obtained about previous illness in 87 cases. In eight instances there had been definite illness within a year: malaria, 3; measles, 2; rubella, 1; mumps, 1; bronchitis, 1. Prior to January, 1918, there had been in addition to the usual diseases of childhood: typhoid, 5; scarlet fever, 4; diphtheria, 2; pneumonia, 6; "grippe," 3. In two cases tonsils had been removed. Two men gave a history of frequent colds in winter. In the group as a whole there was no evidence of respiratory tract ailments just prior to the outbreak.

Meals were served to these men in a separate building several blocks away from their dormitories. Mess-kits were not used. The food and general supervision were the same as for the rest of the

student community including Section A of the S. A. T. C. The men had nothing to do with the washing of dishes and tableware which are known to have been thoroughly cleansed in boiling water.

During the epidemic period another group (Section A) of the Student Army Training Corps were likewise under observation. The majority of this group (685) were housed in dormitories and fraternity buildings under conditions very similar to those obtaining in Section B. Their class rooms and eating places were entirely apart from those of Section B, and the men of the two groups came into no sort of formal contact with one another. The cases of influenza among 685 men in Section A occurred as follows:

Week Ending	Cases in Section A
Oct. 5.....	7
Oct. 12.....	10
Oct. 19*.....	7
Oct. 24.....	4
Nov. 2.....	2
Nov. 9.....	3

*Week of maximum prevalence in Chicago.

The number affected in the different dormitories used by Section A is as follows:

	Approximate Number of Men	Number of Cases Influenza
H	215	14*
S	120	6
M D.....	50	4
N D.....	100	4
S D.....	100	3
P	100	2
	<hr/> 685	<hr/> 33†

* These cases developed on well-scattered dates between October 6 and November 8, never more than two in one day.

† Two of these cases developed pneumonia; there were no deaths.

In addition to this number there were 271 men of Section A living in barracks—half of this number after October 20, the other half after October 29; only two cases of influenza developed in this group, both on the same day (November 8). At the time these men entered barracks influenza in Chicago had decreased considerably from the maximum. Beginning with the assembling of the students, October 1, and throughout the epidemic period, special care was taken to detect cases of incipient illness. Frequent talks were given to men and officers and all men of Section A with any sign of illness, objective or

subjective, were instructed to report to the medical officer, and whether "simple colds" or suspected influenza, were at once isolated in the hospitals or sent to their homes. During the whole period lectures and other classes were held as usual, one group of 350 men meeting three times a week.

The groups may be compared as follows:

	Section A	Section B
Number of men.....	685	234
Cases influenza developing October 17-22.	2	92
Total cases influenza developing October 17 to November 8.....	26	93*
Cases pneumonia	2	12
Deaths	0	2

*In nine others the exact date of onset could not be ascertained.

The mode of housing was similar in the two groups; the food supply was under central supervision and the men themselves had nothing to do with its preparation or serving; neither group received any specific or mixed influenza vaccines. The cases of illness that developed in Section A were quickly isolated, whereas in Section B isolation was less early and much less complete.

Cessation of influenza in Section B followed immediately after the isolation of all cases and the inauguration of daily inspection. The natural immunity of the men of Section B who had not become infected before October 21 was undoubtedly relatively high.

A third group of students, men and women, not living in barracks nor for the most part in dormitories, but at their own homes or in boarding houses gave the following record:

Number of students.....	82
Cases of influenza.....	7
Cases of pneumonia.....	1

The case incidence is here somewhat higher than in Section A where the greater restrictions placed on individual movement unquestionably decreased the amount of contact with the civilian community. The general degree of health supervision was also less than in the Student Army Training Corps unit.

Comparison of these two groups (Section A and civilian students) with the heavily affected Section B, in which the case incidence was about six times as great, indicates the importance of early detection and isolation of influenza cases as a preventive measure.

II. THE UNIVERSITY HIGH AND ELEMENTARY SCHOOLS

EDWIN O. JORDAN AND E. B. FINK

The University of Chicago, through its School of Education, maintains an elementary and a high school. In the office of the Director of Physical Education, careful records of all illnesses among students are kept. We are indebted to Dr. W. J. Monilaw, Physical Director, for opportunity to use his admirable records. Whenever a student is absent from class the teacher fills out a form slip and reports to the school physician. Each day the office secretary makes telephone inquiries as to the causes of absences. The information obtained from the family and the attending physician is recorded on the same form. Students returning after absences are required to report to the office of the school physician for examination. Cases of illness developing during school hours are always examined for the detection of contagious diseases, a woman physician being in attendance for girls. A permanent daily record of all illnesses by classes and cause of illness is kept on file.

The data contained in these records have furnished an opportunity for an epidemiologic study of influenza during the autumn quarter, 1918, as it affected a select group of individuals. The student body consists of boys and girls in the immediate neighborhood of the University, many of them from the families of members of the University faculties. The clientèle of the school is such that physicians are more likely to be consulted for minor illnesses than is the case with children in public schools.

Elementary School.—The autumn quarter began October 1 and ended Dec. 20, 1918, covering a period of approximately 12 weeks. At the beginning of the quarter there were registered 391 pupils, of whom 199 were boys and 192, girls, the youngest being 4 years and the oldest 13 years old. Ninety-seven (97) cases of influenza were reported, a morbidity rate of 24.8 per cent. There were 50 cases among boys and 47 among girls.

Table 1 shows the distribution of illness by months according to grades together with the number and ages of the pupils in each grade. Under the heading, colds, are included "pharyngitis" and "laryngitis" (9 of pharyngitis and 26 of laryngitis). Combined in age groups 4-9 (137 pupils) and 9-14 (229 pupils) the former had 42 and the latter 55 influenza cases, making the attack rate higher in the younger

TABLE 1

CASES OF ILLNESS DEVELOPING IN ELEMENTARY SCHOOL BY MONTHS AND GRADES FROM SPECIFIED CAUSES DURING AUTUMN QUARTER, 1918

Class	Number of Pupils*	Ages	Influenza			Colds			Nonrespiratory		
			Oct.	Nov.	Dec.	Oct.	Nov.	Dec.	Oct.	Nov.	Dec.
Kindergarten.....	32	4-6	2	2	6	8	12	5	7	2	1
1.....	24	6-7	3	4	9	5	33	4	5	6	6
2.....	51	8	6	2	3	19	11	6	12	1	7
3.....	30	8-9	1	2	2	11	8	6	3	1	4
4.....	57	9-10	2	10	4	16	13	11	2	7	1
5.....	58	10-11	4	4	5	10	10	8	15	6	4
6.....	58	11-12	8	8	..	19	10	10	5	10	9
7.....	56	12-13	5	3	2	16	15	5	11	8	6
Totals.....	366	4-13	31	35	31	104	112	55	60	41	38

* Blank space indicates no cases. "Colds" includes respiratory infections other than influenza such as pharyngitis, laryngitis, and bronchitis. "Nonrespiratory" includes headaches, accidents, intestinal disturbances, etc. "Number of Pupils" are those on the rolls in the middle of the autumn quarter.

TABLE 2

DATE OF DEVELOPMENT OF INFLUENZA BY WEEKS, AUTUMN QUARTER, 1918, AS COMPARED WITH COLDS, AUTUMN QUARTER, 1918 AND 1917
Elementary School

Class		Week Ending												Total	
		October				November					December				
		5	12	19	26	2	9	16	23	30	7	14	21		28
Kindergarten	Influenza.....	1	..	1	1	1	3	2	1	..	10
	Colds.....	1	2	2	2	1	2	5	3	1	3	2	24
1	Influenza.....	2	1	1	..	1	..	2	3	1	5	..	16
	Colds.....	..	1	2	2	..	4	6	1	2	1	2	1	..	22
2	Influenza.....	3	2	1	2	3	11
	Colds.....	4	6	4	4	2	1	1	3	..	3	3	30
3	Influenza.....	1	..	1	1	1	..	1	..	5
	Colds.....	2	5	3	1	3	2	2	4	1	1	..	24
4	Influenza.....	..	2	1	..	2	7	2	..	2	..	16
	Colds.....	3	7	3	1	3	6	3	5	4	2	..	37
5	Influenza.....	2	..	1	1	2	1	1	2	2	1	..	13
	Colds.....	3	3	2	2	1	..	5	2	4	2	4	2	..	30
6	Influenza.....	4	3	..	1	1	3	4	16
	Colds.....	5	6	3	3	2	4	3	5	5	36
7	Influenza.....	3	1	1	3	1	1	10
	Colds.....	2	4	0	1	3	..	2	2	..	4	..	1	..	19
Totals:	Influenza.....	13	8	7	3	3	3	4	6	19	15	6	10	..	97
	Colds.....	20	34	19	15	7	7	27	23	15	27	21	7	..	222
	Colds, 1917.....	15	23	17	12	12	15	21	10	6	15	19	5	1	171

Note: During the autumn quarter, 1917, there were 2 cases of "grip" reported in the elementary school—1 with an illness of 6 days, the other, 11 days.

children (307 against 240). Compared by months, November showed the largest number of cases, and of these there were more in the kindergarten and Grade 6 B than in the other classes. The month of highest incidence of colds corresponds with the month of greatest prevalence of influenza and the age and class distribution is about the same; the 7th grade pupils varying in age from 12-13 had as many colds as the 4th grade, both being highest with 44 cases each.

A comparison of the number of days illness due to influenza and colds shows that in October, 31 cases of influenza caused 555 days illness, as against 78 cases of colds with an illness of 520 days; in November the ratio was 35 cases of influenza with 502 days illness and 78 colds with an illness of 499 days; in December, 31 cases of influenza resulted in 494 days illness compared with 60 cases of colds

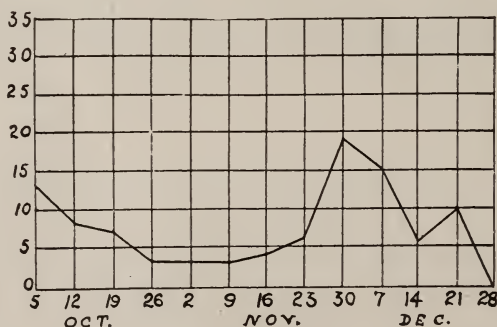


Chart 1.—Showing date of development of cases of influenza, elementary school, autumn quarter, 1918, by weeks.

and 360 days illness. The average period of illness was more than twice as long in influenza as in colds. The number of days of illness caused by influenza is 555 as compared with 825 days from all other causes during October, 502 to 816 in November and 494 to 502 in December. There were 12 instances in which 2 children, and 1 in which 3 children in the same family were reported ill with influenza. One girl, 7 years old, had 3 attacks of so-called influenza, and 1 boy, 5 years old, had 2.

Table 2 and Charts 1-3 show the development of cases of influenza by weeks during the autumn quarter, 1918, and of colds for the same period and for the corresponding quarter, 1917. During the first week of school in 1918, 13 cases of influenza were reported. Following this there was a gradual decline extending over two weeks, to the level

of 3 cases where it remained for 3 weeks, then in the 2 weeks following went up to 4 and 6, respectively, and suddenly during the week ending November 30 jumped to 19 cases, followed by a decline extending over 3 weeks to 10 cases during the last week of the quarter. The epidemic was characterized by a moderate outbreak during the first week of school, followed by a decline extending over 2 weeks to a low level, which was maintained for 5 weeks and then a sudden peak reaching the highest point in the epidemic during the week ending November 30, followed by a decline extending over 3 weeks. While the height of the epidemic of influenza in the city at large was reached during the week ending October 26, as shown by the mortality from influenza and pneumonia, the epidemic among this particular school population did not reach its highest level until the week ending

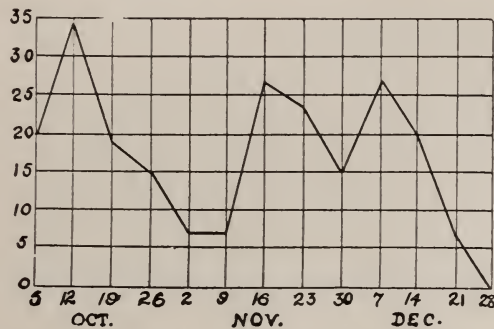


Chart 2.—Showing date of development of colds (other respiratory infections), elementary school, autumn quarter, 1918, by weeks.

November 30. During the week of maximum mortality in Chicago, the number of cases developing among these school children was low, the maximum being reached 5 weeks later. Whether, as is probable, this has any relation to the prevalence of influenza in the portion of the city where the school is located is not certainly known; it was noted in general that the epidemic did not develop in certain districts of the city until after it had subsided in others.

Chart 2 shows that colds in 1918 rose to a high point during the first week, reaching their highest level during the second week, followed by a sharp decline to the lowest level at the fifth week, where they remained 1 week, to rise sharply to a second peak at a lower level than the first one, followed by another sharp decline over 2 weeks to about one-half the lowest level, and in the next week a third peak to

about the level of the second followed by a rapid decline in the last two weeks of school.

A comparison of the curves for influenza and colds shows that the period of highest incidence of colds was in the second week of school and preceded the corresponding period for influenza by 7 weeks. There are 3 peaks in the curve for colds, and only 2 in that for influenza. The period of highest incidence of colds follows the first peak in the influenza curve by 1 week, while during the week of greatest prevalence of influenza there is a sharp fall in the number of cases of colds. The third peak in the curve for colds occurs just 1 week after the height of the influenza curve. The curve for colds as a whole runs at a higher level than that for influenza. A striking thing is that the portion of the curve for influenza contained within

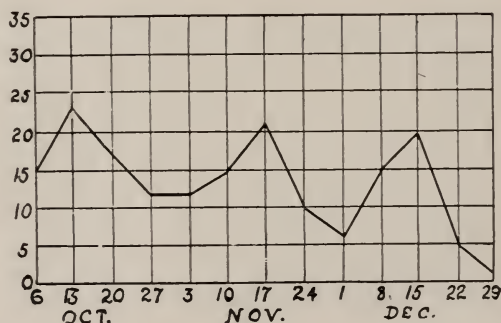


Chart 3.—Showing date of development of colds (other respiratory infections), elementary school, autumn quarter, 1917, by weeks.

the period November 23 to December 7 is almost the exact opposite of the corresponding portion in the curve for colds. How much of this is due to the factor of diagnosis is difficult to say.

The curve for colds in the autumn quarter, 1917, has in general the same outline as the one for 1918. (Chart 3.) It, too, contains three peaks, the first two occurring at exactly the same time, the last a week later. Colds in 1918 were at a higher level than in 1917. The figures for 1917 and 1918 are closely comparable, since they deal largely with the same individuals. About 100 pupils leave school annually, about 50 graduating. The majority of new pupils enter the kindergarten, the rest replace children who have moved or leave for various reasons. The exact number of pupils in 1917 is 380 compared with 391 in 1918, about 300 being the same in both groups.

There are 21 teachers in the elementary school, and among these 5 cases of influenza were reported. Two occurred in the second week of October, 2 in the second week of December and 1 in the fourth week of December, with a total illness of 61 days. Two were second grade teachers, one a substitute, one special and one a teacher of physical culture. There were no complications and no deaths. As far as could be determined there seemed to be no evidence that any of the teachers acted as a focus of infection.

Of the 97 cases of influenza reported among the grade school pupils, none developed pneumonia, and there was no death.

TABLE 3

COMPARATIVE NUMBER OF CASES OF INFLUENZA AND COLDS, AUTUMN QUARTER, 1918 AND 1917,
BY WEEKS AND SEX. HIGH SCHOOL

	Week Ending													Totals
	October				November					December				
	5	12	19	26	2	9	16	23	30	7	14	21	28	
Influenza, 1918:														
Boys.....	6	5	..	1	..	1	2	1	8	4	6	5	2	41
Girls.....	17	3	4	4	1	1	2	..	8	1	5	3	1	50
Totals.....	23	8	4	5	1	2	4	1	16	5	11	8	3	91
Colds, 1918:														
Boys.....	8	6	10	7	3	1	1	1	2	13	11	7	6	76
Girls.....	7	7	10	6	4	3	4	6	13	21	17	10	3	111
Totals.....	15	13	20	13	7	4	5	7	15	34	28	17	9	187
Colds, 1917:														
Boys.....	2	4	13	5	..	9	5	8	3	10	3	62
Girls.....	1	5	1	5	4	4	6	5	6	8	6	5	..	56
Totals.....	1	5	3	9	17	9	6	14	11	16	9	15	3	118

Note: In 1917, 2 cases of "grip" were reported, 1 with an absence of 4 days, the other 1 day.

High School.—At the beginning of the autumn quarter, 1918, there were 427 students registered in the high school, of whom 199 were boys and 228 girls. In age, they varied from 14-18 years. Many of the children graduating from the elementary school continue in the high school. Ninety-one cases of influenza were reported, a case incidence of 21.3%. A slightly larger number occurred among girls, the exact ratio being 41 for boys and 50 for girls, making the attack rate approximately the same for the two sexes.

During the same period there were 189 cases of colds as against 118 for the corresponding period in 1917.

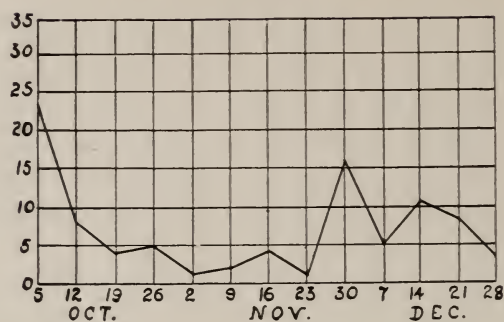


Chart 4.—Showing date of development of cases of influenza, high school, autumn quarter, 1918, by weeks.

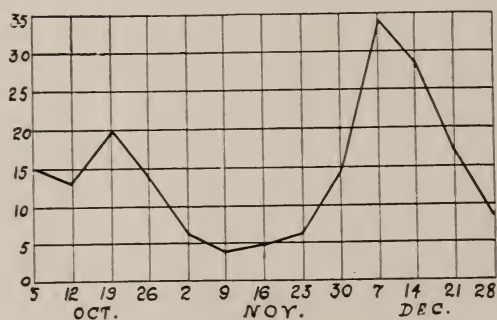


Chart 5.—Showing date of development of colds (other respiratory infections), high school, autumn quarter, 1918, by weeks.

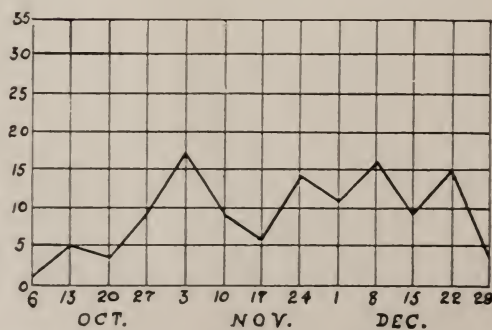


Chart 6.—Showing date of development of colds (other respiratory infections), high school, autumn quarter, 1917, by weeks.

Chart 4 illustrates graphically the curve of influenza by weeks. The week of highest incidence was the first week of school with 23 cases. This was followed by a sharp drop over a period of 2 weeks to a low level. A second peak occurred during the week ending November 30 with 16 cases, after which the epidemic rapidly subsided. A comparison with the course of influenza in the elementary school shows that in the high school the epidemic was most marked in the beginning, the secondary outbreak being less severe. The reverse was true of the elementary school. Both peaks occurred at the same time. The curve for influenza in the high school is at a slightly higher level than in the elementary school. The curve for colds is irregular. (Chart 5.) There are two peaks, the first occurring during the week when influenza had subsided after the first outbreak, the second a week following the second outbreak. Both correspond with sharp falls in the influenza curve. Colds in 1917 (Chart 6) followed a much more irregular course, never reaching the maximum height during 1918.

In October, 40 cases of influenza resulted in a loss of 331 school days as compared with 76 cases of other respiratory infections, including colds, which caused a loss of 236 school days and 72 cases including all nonrespiratory illnesses with an absence of 118 days. In November, 24 cases of influenza resulted in a loss of 118 school days, 46 cases of other respiratory diseases, 163 days, and all others combined totaled 59 cases and 112 days absence. In December, 27 cases of influenza resulted in an absence of 188 school days, other respiratory diseases 94 cases with a loss of 187 school days, while all other illnesses were 66 cases and 90 days lost. Almost as many school days were lost in October through influenza as from all other causes combined; during November the ratio was less than one-half and during December about two-thirds.

All cases of influenza recovered. Two cases of pneumonia were reported, both in girls. These were carefully investigated. One apparently started as a bronchitis while the other undoubtedly began as a severe influenza with chills, high temperature, prostration and general muscle pains. On account of its severity this case was diagnosed as pneumonia from the beginning.

Out of a total of 42 teachers in the high school, 6 developed influenza and all recovered.

An interesting feature of the figures given above is the rise in influenza cases in both the high and elementary schools about Novem-

ber 30, following the Thanksgiving recess from Wednesday to Monday. The parties and family gatherings at that time apparently afforded a better opportunity for influenza infection than the routine school life before and after the holiday period.

All the facts gathered afford no evidence that the schools served as distributing points for influenza infection.

III. THE CHICAGO TELEPHONE COMPANY

EDWIN O. JORDAN AND E. B. FINK

The Chicago Telephone Company maintains a sickness benefit system to which all employees who have been with the Company for a period of two years or more are eligible. For administrative purposes complete records of all cases of illness developing among employees entitled to benefits are kept in the Company's health department. Examination of these records has enabled us to determine the course of the influenza epidemic in Chicago in an occupational group of the adult population. We are greatly indebted to Mr. S. J. Larned for opportunity to examine these records and to Mr. H. W. Bang, Miss K. O'Rourke and Miss K. Ryan for valuable aid in assembling the data.

The figures cover the period from Sept. 1, 1918, to the middle of March, 1919, or 26 weeks. In January, 1919, which represents about the middle of the period covered, the Chicago Telephone Company had in its employ 14,208 individuals, 3,927 males and 10,281 females. Of the total number of employees, 53%, or 7,530, were eligible to sickness benefits. Approximately, 80% of the men, or 3,141, are entitled to disability benefits; and 40%, or 4,112 of the women. This difference is the natural result of the type of work in which the two sexes are engaged. The work done by the men is a specialized type of skilled labor, and the turnover is small. Shifting of the women workers is much more frequent.

The data include approximately 7,500 individuals of working age. Among these there developed a total of 1,448 cases of influenza (including "la grippe") during the period under consideration, an attack rate of 19.2%. There were 22 deaths attributed to influenza and influenza-pneumonia, a mortality of 1.5%.

Table 4 and Chart 7 illustrate the development of influenza cases by weeks. The epidemic was characterized by two distinct peaks. The first and higher began the third week in September, and shot up to a maximum of 180 cases for the week ending October 12, approximately four weeks after the beginning of the epidemic. The decline of the first wave was almost as sharp as its beginning and extended over a period of five weeks, reaching its lowest level during the week ending November 16. The rise of the second wave also covered a period of four weeks, reaching its maximum in the week ending December 14. The maximum number of cases developed during this week was 78, or less than one-half the height of the first peak. Following the second peak there was an irregular, but gradual decline extending over a period of 12 weeks.

TABLE 4

DATE OF DEVELOPMENT OF CASES OF INFLUENZA AND COLDS (ALL OTHER RESPIRATORY INFECTIONS) DURING THE PERIOD SEPT. 1, 1918, TO MARCH 15, 1919, BY WEEKS
CHICAGO TELEPHONE CO.

Week Ending	Influenza	Colds	Week Ending	Influenza	Colds
Sept. 7.....	2	2	Dec. 14.....	78	19
14.....	3	3	21.....	49	13
21.....	26	5	28.....	41	18
28.....	58	13	Jan. 4.....	54	28
Oct. 5.....	134	11	11.....	49	18
12.....	180	14	18.....	40	18
19.....	176	25	25.....	39	20
26.....	105	22	Feb. 2.....	26	15
Nov. 2.....	71	7	9.....	29	19
9.....	38	9	16.....	30	12
16.....	20	10	23.....	23	22
23.....	25	4	Mar. 1.....	25	25
30.....	38	6	8.....	19	24
Dec. 7.....	66	17	15.....	4	3
Total.....				1,448	402
Minimum.....				2	2
Maximum.....				180	28

During the same period there was a total of 402 colds (including all other acute respiratory infections except influenza). Chart 8 shows the development of these cases by weeks. The curve follows an irregular course at a low level with only two distinct peaks — the first, one week after the maximum week of influenza and the second, three weeks after a similar peak in the curve for influenza. During the corresponding period in the preceding year, 1917-18, a nonepidemic year, there was a total reported of 300 cases of "influenza" and "la grippe," and 219 colds.

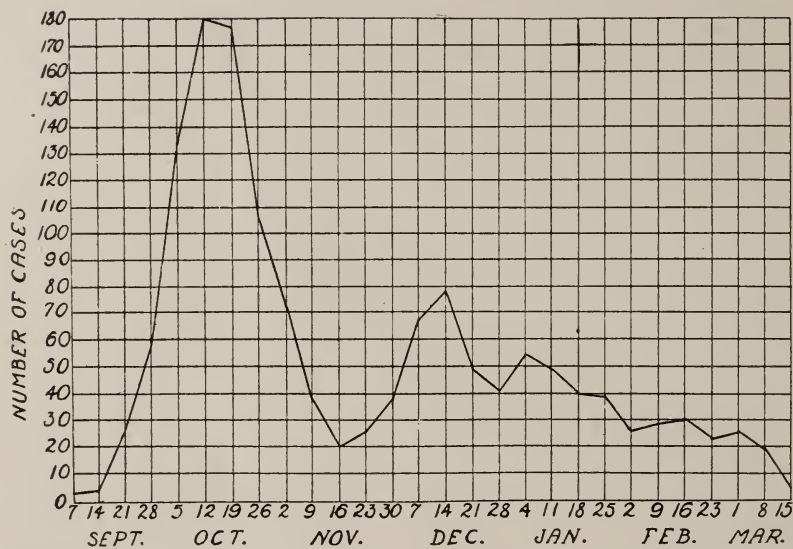


Chart 7.—Showing date of development of influenza cases, Chicago Telephone Co., Sept. 1, 1918, to March 15, 1919, by weeks.

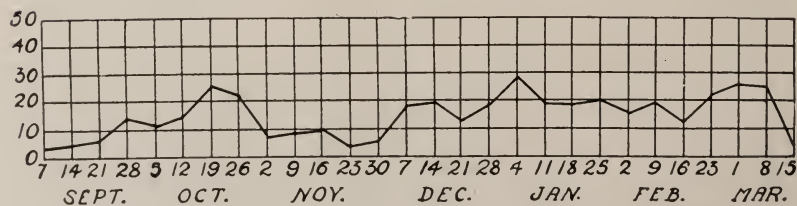


Chart 8.—Showing date of development of colds (other respiratory infections), Chicago Telephone Co., Sept. 1, 1918, to March 15, 1919, by weeks.

A comparison of the number of days lost on account of influenza and colds indicates that in 1,177 cases of influenza about which we were able to obtain a record of the period of disability, a total of 27,154 working days was lost, while 346 colds resulted in a loss of 7,374 days. The average number of days lost per case of influenza was 23, and of colds, 21. During six month, September, 1918, to February, 1919, inclusive, there was a total loss of 79,253 working days from illness of all kinds; influenza was responsible for 34% of the total loss. A comparison of the total number of days disability in 1917 and 1918 by months shows that during October, 1918, the month during which the influenza epidemic was at its maximum, there were more than twice as many days lost as in October, 1917. The effect of the entire epidemic is reflected in the sudden rise in disability for the corresponding months. During March and April, 1918, there was an epidemic of "la grippe" (a total of 409 cases), and during these months the total sickness disability reached nearly the high level of the recent influenza epidemic.

Table 5 presents the result of an analysis of 1,432 cases of influenza as to age and sex distribution. About 80% of the male employees of the Company are between the ages 20 and 35. Eighty per cent. of the women are between the ages 17 and 25, both inclusive. The majority of cases among women occurred within the age groups 16 to 30; among men, 26 to 40. Considering the age distribution of all the employees of the Company, the figures indicate that the cases of influenza were fairly evenly distributed in proportion to the number of people exposed at the different age groups. A total of 957 cases occurred among females; 475 among males, making the attack rate among the women 23%; among the men 15%.

TABLE 5
AGE AND SEX DISTRIBUTION OF INFLUENZA CASES, CHICAGO TELEPHONE CO.

Age Group	Males	Females	Age Group	Males	Females
16-20	8	200	41-45	41	8
21-25	37	447	46-50	11	5
26-30	128	220	51-55	3	1
31-35	150	56	56-60 and over	8	2
36-40	89	18			

The histories of 218 cases of influenza were examined to determine the most common symptoms and complications. Each record contained the certificate of a physician as to the diagnosis, complications and prognosis, the report of the visiting nurse, including pulse, temperature, respiration and general symptoms at the time visits were made, usually at 3-day intervals, and a report of the findings of the Company physician when the patient was ready to return to work.

The most frequent symptoms were high temperature, 101-103 F., weakness and prostration, pain in the back, headache, aches "all over." Occasionally epistaxis and profuse bleeding from the mouth were mentioned. Neuralgia of the face was complained of in one or two cases. The onset in some cases was characterized by chills, fever and cough; others began as a cold, sore throat and watering of the eyes and nose. In some cases the patients fainted while at work. On examination by the Company physician after recovery, inflamed tonsils were frequently found.

Table 6 shows the complications developing in the course of 218 cases of influenza. One hundred and fifty-seven, or 72%, were uncomplicated. The most frequent complication was bronchitis. A distressing complication occurring in 5 cases was a prolonged convalescence or postinfluenzal neurasthenia. Sinus infections occurred in about 2% of cases and otitis media in 1%. Pneumonia was diagnosed in 17 cases, or 7.8%. There were 2 deaths, a mortality of less than 1%.

TABLE 6
COMPLICATIONS IN 218 CASES OF INFLUENZA, CHICAGO TELEPHONE CO.

No recorded complications.....	157	Tonsillitis.....	4
Bronchitis.....	45	Gastritis.....	4
Pneumonia.....	17	Pleurisy.....	3
Relapses.....	9	Otitis media.....	3
Neurasthenia.....	5	Miscellaneous.....	12
Sinus infection.....	5		

An analysis of the records of the social service department has enabled us to determine the proportion of girls at the different telephone exchanges who were attacked with influenza. The data include all the employees at the same exchange—those entitled to disability benefits as well as those not entitled. In the case of those entitled to benefits the diagnoses and complications were based on physicians'

certificates as well as visiting nurses' records; the remainder were based on nurses' records alone where no physician was in attendance. The nurses' records included general symptoms as well as observations on temperature, pulse and respiration.

Table 7 shows the number of employees at each exchange, the number who had influenza during the period, September, 1918, to February, 1919, inclusive, and the attack rate. (Chart 9.) The total number of cases at all exchanges was 1,072, the total number of employees concerned was 7,804 (as of January, 1919), giving an attack rate of 13.6%. The highest percentage attacked by influenza at any exchange was 27, the lowest 3. It was noted by the administrators of the social

TABLE 7
PROPORTION OF TELEPHONE OPERATORS AT THE DIFFERENT EXCHANGES—CHICAGO TELEPHONE COMPANY—COMING DOWN WITH INFLUENZA DURING THE PERIOD, SEPTEMBER, 1918, TO FEBRUARY, 1919, INCLUSIVE

Exchange	Number of Girls at Each (Jan. 1919)	Rate of Attack	
		Number	Per Cent.
Austin.....	170	46	27
Belmont.....	178	28	16
Beverly.....	111	21	19
Burnside.....	11	1	9
Calumet.....	206	26	12
Canal.....	126	7	5
Central.....	549	52	9
Douglas.....	153	22	14
Edgewater.....	312	59	19
Humboldt.....	248	49	20
Hyde Park.....	348	46	13
Irving.....	177	36	21
Kedzie.....	265	31	11
Kildare.....	67	10	15
Lake View.....	352	52	14
Lawndale.....	221	35	16
Lincoln.....	180	32	18
Main.....	586	80	13
McKinley.....	86	15	17
Monroe.....	276	13	5
Oakland.....	487	66	13
Pullman.....	91	13	14
Rogers Park.....	108	18	17
South Chicago.....	126	4	3
Stewart.....	140	14	10
Superior.....	186	31	17
Wabash.....	598	72	12
Wentworth.....	335	63	19
West.....	171	14	8
Yards.....	274	31	11
Toll.....	416	61	14
Operators training.....	40	5	12
Pay station.....	110	10	9
Traffic department.....	100	9	9
Totals.....	7,804	1,072	13.7
Total pneumonia cases.....		71	6.6%
Total deaths.....		10	1.0%

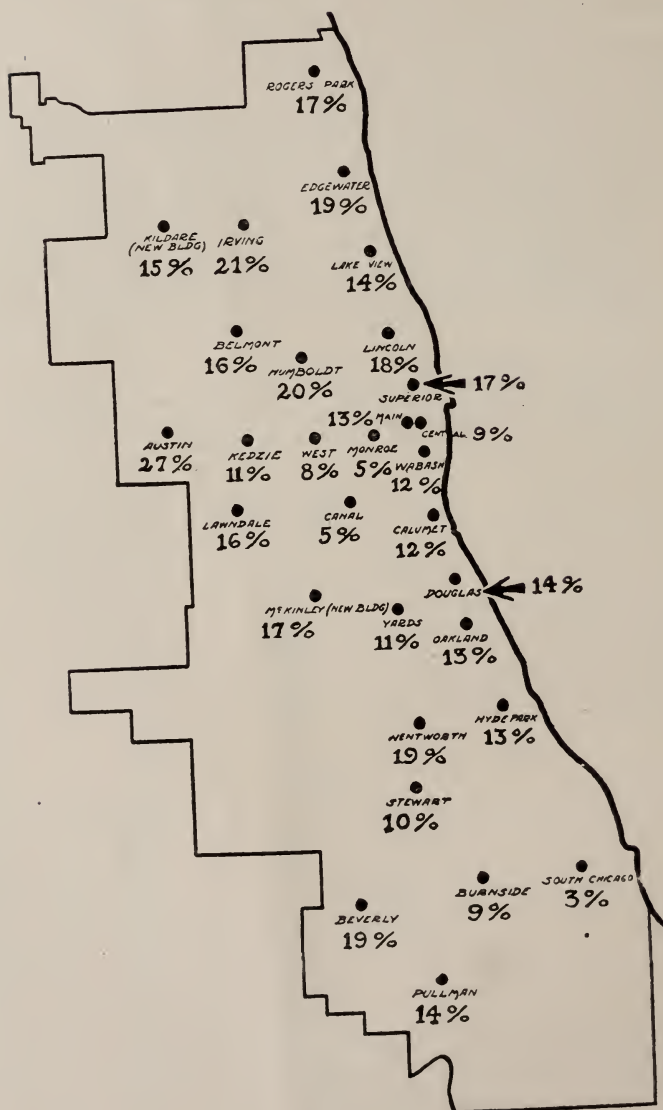


Chart 9.—Map showing location of telephone exchanges and proportion of girls at each exchange attacked by influenza. Chicago Telephone Co.

service department that the largest number of cases developed first on the northwest side, and it was not until about one month later that the epidemic reached the south side of the city. There were 71 cases of pneumonia recorded among the 1,072 cases of influenza, or a rate for this complication of 6.6%. Only 10 deaths were reported, a mortality rate of less than 1%. An interesting fact bearing on the source of infection noted in this series of cases as well as among those entitled to disability benefits, was that in a great many cases the record was made by the nurse that several or all other members of the family were ill with influenza. Frequently one or more deaths were reported among other members of the family. It is not considered worth while to record the exact number of times such an observation was made because the record of such family infection is manifestly incomplete.

SUMMARY

The data for the several groups may be brought together as follows:

TABLE 8
GENERAL SUMMARY OF OBSERVATIONS

Group	Number of Individuals	Influenza Attack Rate per 1,000	Case Fatality Rate	Proportion of Clinically Diagnosed Pneumonia to 100 Influenza Cases
1. Student Army Training Corps, Section A	685	39	0	7.7
2. Student Army Training Corps, Section B	234	398	2	13.0
3. Pupils, Elementary School, University of Chicago.....	291	248	0	0.0
4. Pupils, High School, University of Chicago	427	213	0	2.2
5. Teachers, Elementary and High Schools	63	175	0	0.0
6. Chicago Telephone Co. Employees Eligible to Disability Benefits...	7,530*	192	1.5	7.8†
7. Chicago Telephone Co. Women Employees at Exchanges.....	7,804	137	1	6.6

* Includes about 40% of Group 7.

† Based on 218 cases.

With respect to age, the figures show a higher attack rate among the pupils of the university elementary school (ages 4-13) than among those of the high school (ages 14-18); the teachers in these schools had a lower attack rate than the pupils. Apparently a definite age incidence is manifested since the pupils in these schools are from the

same section of the city and to a large extent from the same families, and were presumably exposed in similar degree.

With respect to sex, there was no noteworthy difference among the pupils in the high and elementary schools (attack rate—230 for boys, 231 for girls). It is fair to assume that the chances for acquiring infection were substantially the same for these children, and that one sex was as much exposed to infection as the other. Among the employees of the Chicago Telephone Company, on the other hand, the men were affected in considerably lower proportion than the women (151 per 1,000 for men, 233 for women). Probably the age factor was largely responsible for this difference, since the women employees are of a much lower average age than the men.

Illness reported under the heading of "colds," etc., seems to have been at a considerably higher level during the autumn of 1918 than during the corresponding period of 1917. This was particularly the case among the pupils of the university schools and to a somewhat lesser degree among the employees of the Chicago Telephone Company. Comparison of the reported cases of influenza and colds in the latter group for the months September-November suggests that some cases of influenza were reported under the former heading.

The differing degrees of incidence in the various groups here considered are especially striking. The attack rate among the employees at the various Chicago telephone exchanges ranged from 30-270 per 1,000, altho the working conditions in the several exchanges were not materially different. The highest attack rate recorded for any group occurred among members of one section of the S. A. T. C. at the University of Chicago (398), while the lowest (39) was among the members of the other section of the same corps. The former group was particularly exposed to infection, while the latter, altho composed of men of similar ages living under substantially similar conditions with those of the first group, were guarded to a considerable extent against contact with beginning cases.

The data obtained in regard to the schools apparently indicate that the schools were not important distributing centers for the infection. No explosive outbreak occurred in any one grade, and the four days

of the Thanksgiving holiday evidently afforded more favorable opportunities for infection than did the days of regular school attendance. The low pneumonia incidence and the absence of deaths among the pupils of these schools (188 cases) is noteworthy.

The influence of careful supervision of a somewhat segregated group of individuals is shown by the low attack rate in Section A of the S. A. T. C.

THE ANTIGENIC PROPERTIES OF PROTEOSES

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INTRODUCTION

Of fundamental importance in immunity is the question whether only proteins are antigenic. Wells¹ says, "as yet, it has not been finally established that any colloids other than proteins can act as antigens." Assuming then, that only proteins possess antigenic properties, it becomes of interest to inquire what there is in the nature or constitution of proteins to which this is due. Two characteristics of proteins have received the greatest attention in this connection; their colloid nature and their huge molecular weight. Attempts to explain the antigenic properties of proteins on the basis of their colloid nature have been largely speculative and consist of the application of the principles of colloid chemistry to theories of immune reactions. The efforts to determine the influence of the size of the molecule on the antigenic properties of proteins have led to numerous attempts to produce antibodies for the products of protein hydrolysis and digestion.

Our understanding of the chemistry of immune reactions is limited by our knowledge of the chemistry of proteins. Each new insight into the nature and constitution of proteins is followed by a readjustment of our theories of immuno-chemistry. Underhill and Hendrix² place the discovery of the phenomenon of anaphylaxis among the more important recent advances in the physiology of proteins. It has also furnished considerable impetus to the study of protein chemistry.

For a satisfactory explanation of the anaphylaxis reaction, it became necessary to determine by what changes proteins were rendered toxic, since it was well known that native proteins were of themselves nontoxic. This led to the theories of Vaughan, Friedmann and Friedberger that anaphylaxis is due to parenteral digestion of proteins, and that the symptoms are due to intoxication with the resulting products. To obtain evidence in support of these theories the products of protein

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¹ Chemical Pathology, 1914, p. 156.

² Jour. Biol. Chem., 1915, 22, p. 443.

digestion and cleavage have been injected into animals in attempts to produce the symptoms of anaphylactic shock.

Considerable work has been done by many investigators in an effort to demonstrate the formation of antibodies against incomplete protein molecules. Most of the attempts have been made with the higher cleavage products of protein digestion.

HISTORICAL

(a) *Anaphylaxis and "Peptone Shock."*—Evidence is not lacking that the higher products of protein disintegration may act as antigens. The most successful results have been reported with the anaphylaxis reaction.

Rosenau and Anderson³ were among the first to ascribe anaphylactogenic properties to "peptone." They state that "peptone" seems to have slight sensitizing and intoxicating properties but the table showing their results indicates that 0.004 gm. was injected subcutaneously into a guinea-pig, followed 31 days later by a like amount which produced no symptoms of anaphylactic shock. They fail to mention the source or kind of "peptone" used.

Arthus⁴ studied the anaphylaxis reactions of proteoses as represented by commercial Witte peptone. His first experiments were on dogs. He noted a striking resemblance between the reaction of sensitized animals to a second injection of serum and the reaction of normal dogs to an injection of "proteoses." He was able to increase the toxicity of Witte peptone by repeated subcutaneous injections; this he interprets as a hypersusceptibility to proteoses. Experiments with rabbits yielded practically the same results. In a sensitized rabbit (to horse serum) a dose of "peptone" which produced no effect in a normal animal caused a marked fall in blood pressure, dyspnea and loss of sphincter control.

It is doubtful if any of the reactions described by Arthus following the injection of Witte peptone represent true anaphylaxis reactions. Zunz⁵ has pointed out that it is not at all surprising that conflicting results have been obtained by the use of Witte peptone, since its composition is extremely variable, especially the proteoses contained in a given sample. Much emphasis was laid on changes in respiratory rate in "sensitized" rabbits, whereas mere handling of these animals is often sufficient to produce marked dyspnea and tachycardia.

Pick and Yamanuchi,⁶ using a 10% solution of Witte peptone from which all coagulable material had been removed by boiling, produced symptoms of anaphylaxis and death in a rabbit sensitized to beef serum. The dose of peptone was 4 cc intravenously. Two young rabbits passively sensitized against Witte peptone died on intravenous injection of 6 cc of 10% heated peptone. Control animals showed no symptoms. In these experiments young rabbits were used, and sensitization was passive.

After reviewing the symptoms of anaphylaxis in the different laboratory animals, Biedl and Kraus⁷ called attention to the fact that the symptom complex as described in the literature up to that time was not at all definite.

³ Hyg. Lab. Bull. 36, U. S. Pub. Health Serv., 1907.

⁴ Arch. Internat. de Physiol., 1909, 7, p. 471; 1910, 9, p. 157 and p. 179.

⁵ Ztschr. f. Immunitätsforsch., 1913, 16, p. 580.

⁶ Ibid., 1909, 1, p. 676.

⁷ Wien. klin. Wchnschr., 1909, 22, p. 363.

They therefore made a careful study of anaphylaxis in the dog because they considered this animal most suitable for the study of physiologic phenomena. All of the dogs used did not respond to a second injection of horse serum. The characteristic symptoms in those that did react was a fall in blood pressure due to peripheral vasodilatation, a loss of coagulability of the blood and a primary leukopenia followed after several hours by a leukocytosis. None of their dogs died of an acute anaphylactic reaction. In comparing these symptoms with the action of Witte peptone, they noted a close similarity between the two. They concluded from their experiments and from review of the literature that the effect of peptone is in all respects similar to the reinjection of horse or beef serum even in minutest detail. They do not say that anaphylaxis is produced by peptones or proteoses but believe that Witte peptone contains the active principle which is responsible for the symptoms of anaphylaxis. As additional evidence they state that dogs were rendered antianaphylactic against serum by the injection of Witte peptone, and conversely animals which had recovered from the effects of a second injection of serum could withstand larger doses of peptone.

In a subsequent paper Biedl and Kraus⁸ state that they were unable to confirm the observations of Arthus⁴ that the symptoms of anaphylaxis in the rabbit are similar to those in the dog. They emphasize the fact that their experiments apply only to dogs. By the injection of cultures of various bacteria they were able to partly reproduce the symptoms of anaphylactic shock in normal dogs. These failed to appear when the cultures were grown on peptone-free medium. They therefore conclude that the symptoms were due to the small amounts of peptone present in the medium. Later the same authors⁹ report the results of experiments on rabbits and guinea-pigs. In their opinion the mechanism of anaphylaxis is different in the dog and in the guinea-pig. The effect in the former is on the smooth muscle of the peripheral vessels leading to vasodilatation, while in the guinea-pig the reaction consists of a tetanic contraction of the smooth muscle of the bronchioles. Rabbits were found unsuited to anaphylaxis experiments because they died from blood pressure changes. Peptone proved to be very toxic for the guinea-pig, but the symptoms were not like those in the dog. After intravenous injection of 0.25 to 0.30 gm. Witte peptone into guinea-pigs they found that the interference with respiration and the physiologic and anatomic changes in the lungs were exactly like those in anaphylactic shock. From these results they conclude that in the guinea-pig as well as in the dog, Witte peptone has the same action as the toxic principle responsible for the symptoms of anaphylactic shock.

In discussing the results of Biedl and Kraus it may be said that altho they selected the dog as being most suitable for physiologic experimentation, anaphylaxis represents a special manifestation of immune phenomena, and it does not follow that an animal especially suited to work in the one field will also prove satisfactory in the other. As a matter of fact, the symptoms which they found characteristic of anaphylaxis in the dog do not support their contention. Changes in blood pressure may be produced by a number of chemical substances. The same is true of delayed coagulability of the blood. As Wells¹⁰ points out, "the results obtained by observing changes in blood pressure in dogs are by no means comparable with results obtained

⁸ Cent. f. Bakteriöl., 1909, 44, Ref., Beiheft, p. 68.

⁹ Ztschr. f. Immunitätsforsch., 1910, 7, p. 205.

¹⁰ Jour. Infect. Dis., 1909, 6, p. 506.

with guinea-pigs, on which most of the work so far reported has been done, since in these animals the symptoms are entirely different from the symptoms in the dog, and much more closely resemble the effects seen in man."

It is not the purpose to review in detail the physiologic properties of proteoses. This has been done by Chittenden, Mendel and Henderson.¹¹ They used purified albumoses obtained by acid hydrolysis and digestion of coagulated egg-albumin. All proteoses of whatever source, produced a fall in blood pressure which varied only in degree, and blood withdrawn even 1 or 2 hours after the injection failed to coagulate for at least 24 hours. To produce the characteristic effect 3 to 5 cg. per kg. of body weight are necessary and the intensity of the reaction depends much more on the rapidity of injection than on the dosage. An animal which has recovered from an injection of proteoses may fail to respond to a second injection. This has been termed "immunity" to proteoses, but might better be spoken of as a refractory state, the explanation for which is unknown.

Biedl and Kraus cite the experiments of Popielski and Pick and Spiro on the physiologic action of Witte peptone. The former ascribes the toxicity of the commercial mixture to a substance precipitable by hot absolute alcohol, containing no cholin. This substance he called "vasodilatin." The latter believed the toxic symptoms due to a hypothetical contaminating substance "peptozyme." Underhill¹² after purifying samples of Witte peptone according to the directions of Pick and Spiro, was unable to detect any loss in toxicity. Using purified proteoses obtained by acid hydrolysis of proteins of both animal and vegetable origin he was able to obtain the typical physiologic reactions of proteoses described by Chittenden, Mendel and Henderson. He concludes that "at present there is no occasion for attributing the physiological effects following the injection of proteoses into the circulation to the presence of contaminating substances derived from animal tissue or elsewhere. . . . No method of 'purification' has been found which will deprive proteoses of their characteristic physiological behavior in the circulation; when the chemical make-up of the proteoses is profoundly altered and they lose their chemical identity, the typical physiological action may also be lost."

It seems probable, therefore, that the symptoms of intoxication following intravenous injection of Witte peptone into dogs are due to proteoses contained in the preparation, and is a physiologic reaction differing fundamentally from anaphylaxis in the absence of the phenomenon of sensitization following a suitable incubation period.

The work of Biedl and Kraus stimulated a great deal of investigation and discussion. Richet¹³ believes that it is scientifically impossible to compare the physiologic action of peptones with the action of anaphylactic substances. Fall in blood pressure is not sufficient to explain all the symptoms (vomiting, profuse diarrhea, agitation, paraplegia, intoxication, psychic blindness, coma, all phenomena coming on with extreme rapidity) in a dog rendered anaphylactic with actino-congestine. Amyl nitrite, which in small doses produces a fall in blood pressure, does not result in any such grave symptoms. Regarding incoagulability of the blood, he finds that in anaphylaxis produced by mytilo-congestine or actino-congestine, there is no appreciable diminution in the coagulability of the blood. The congestines have the general characters of peptones.

¹¹ Am. Jour. Physiol., 1898-9, 2, p. 142.

¹² Ibid., 1903, 9, p. 345.

¹³ Presse Medicale, 1909, 17, p. 249.

Each of the symptoms described by Biedl and Kraus as characteristic of anaphylaxis was studied separately by Salus.¹⁴ He injected intravenously into guinea-pigs a sufficient quantity of magnesium sulphate, sodium citrate or hirudin to produce incoagulability of the blood equal to that in anaphylactic shock without finding any of the lung changes at necropsy. Pure preparations of pepsin were nontoxic for guinea-pigs, only those preparations being toxic which contained albumoses as impurities. The albumoses themselves varied in toxicity. Later he¹⁵ attempted to determine whether the principle in anaphylatoxin is a peptone-like, dialyzable ninhydrin-reacting substance. By dialyzing anaphylatoxin he was never able to obtain a toxic substance in the dialysate. According to this anaphylatoxin does not seem to be a peptone-like substance. Horse serum did not sensitize guinea-pigs to peptone, nor did an injection of peptone render an animal refractory to a second injection of horse serum.

According to Doerr and Moldovan¹⁶ the changes in the lungs in anaphylaxis are not specific for this condition but may be produced by such substances as peptone and saponin. Furthermore, they consider it unsafe to speculate on the identity of the toxin in anaphylaxis and a hypothetical substance in Witte peptone which is supposed to produce the symptoms of "peptone intoxication" until the two have been isolated and identified. Witte peptone is a mixture of substances and the toxic principle contained in it has not yet been identified. They evidently were not familiar with the work of Underhill on proteoses.

DeWaele and Vandeveldt¹⁷ observed no symptoms of anaphylaxis following repeated subcutaneous injections of Witte peptone into rabbits. The dosage varied from 0.2-1 gm.

Werbitzky¹⁸ sensitized guinea-pigs with 0.01 gm. horse serum and then injected peptone by various means without producing symptoms except in one animal. The same animals showed the usual reactions of sensitized guinea-pigs when injected with a second dose of horse serum. Peptone did not increase their susceptibility. Doses of peptone which were said to have produced symptoms of anaphylactic shock in dogs (Biedl and Kraus) were found to have absolutely no effect on guinea-pigs. The conclusion is therefore drawn that "peptone intoxication" and protein sensitization are two separate and unrelated phenomena.

The constancy of temperature changes in the guinea-pig during anaphylactic shock is emphasized by Pfeiffer and Mita.¹⁹ There is a fall in temperature often as much as 4 C. They noted a similar drop in temperature after intraperitoneal injection of Witte peptone. By subcutaneous injections of peptone they obtained the Arthus phenomenon or local tissue necrosis at the site of injection. The injection of peptone caused the symptoms to become pronounced in an anaphylactic animal, but peptone was unable to sensitize to itself. The differences between anaphylactic shock and "peptone intoxication" were sufficient to lead them to believe that the two are not identical.

St. Bächer and Wakushima²⁰ made determinations of the opsonic index in the dog during anaphylactic shock and found that there was a marked drop.

¹⁴ Med. Klinik, 1912, 8, p. 1355.

¹⁵ Biochem. Ztschr., 1914, 65, p. 381.

¹⁶ Ztschr. f. Immunitätsforsch., 1910, 7, p. 223.

¹⁷ Biochem. Ztschr., 1910, 30, p. 227.

¹⁸ Compt. rend. de la Soc. de Biol., 1908, 66, p. 23.

¹⁹ Ztschr. f. Immunitätsforsch., 1909, 4, p. 410.

²⁰ Cent. f. Bakteriöl., I, O., 1911, 61, p. 238.

Dogs which received intravenous injections of Witte peptone also showed a marked fall in their opsonic index which varied with the severity of the symptoms.

Friedberger and Mita²¹ injected 0.1-0.5 cc of sheep serum intraperitoneally or into the dorsal sac of frogs. One to four weeks later the animals received an intravenous injection of 0.1 cc of the homologous serum. Following the second injection there were characteristic changes in the heart action. Very soon after injection there was a definite slowing of the rate, marked irregularity, and finally the heart stopped in diastole. These changes are attributed to anaphylactic shock, and the authors point out that definite circulatory changes have been observed in anaphylactic shock in warm blooded animals. The effect of various commercial peptones and a sample of pure silk peptone obtained from Abderhalden was then studied. The peptone mixtures had a toxic effect on the isolated frog heart the intensity of which was much less than that of true anaphylactic shock.

Hirschfelder²² reports that by the intravenous injection of from 5-8 cc of 10% Witte peptone into guinea-pigs he was able to produce the changes in the lungs described by Auer and Lewis as characteristic of anaphylactic shock. This occurred only when the injection was made rapidly. As much as 55 cc intraperitoneally produced no effect.

Manwaring²³ sensitized three dogs against horse serum and after a second injection the animals recovered. They were in no way refractory to Witte peptone but reacted in the usual way. If the mechanism for both reactions were the same we should expect that when animals fail to react against the one they would also fail to react against the other. Manwaring is unwilling to admit that the two phenomena are different, but concludes that the toxic substance formed or set free may be identical in both cases. Loewit²⁴ repeated the experiments of Manwaring, using rabbits and guinea-pigs. He also found that animals which had been made anti-anaphylactic by recovery from a second injection of horse serum reacted to a single injection of Witte peptone in a typical way. So that in the guinea-pig and rabbit as well as in the dog the exhaustion of one mechanism leaves the other still intact.

Nolf²⁵ was able to confirm the findings of Biedl and Kraus in dogs, and also believes that anaphylaxis is identical with "propeptone" intoxication.

Calvary²⁶ found that during anaphylactic shock in the dog there was a lessened flow of lymph which failed to clot. A single injection of protein had no such effect, neither did a mere fall in blood pressure. Witte peptone, on the other hand, had a lymphagogue action. He concludes that if both reactions were the same, their effect on the lymph ought to be the same.

Graetz²⁷ made an extensive study of the anatomic changes in guinea-pigs dying of anaphylactic shock. He found that the circulatory changes and the changes in the lungs were the same as those following a single injection of Witte peptone.

In a study of the blood changes following intravenous injection of egg-white into dogs, Schittenhelm, Weichardt and Grisshammer²⁸ found that the

²¹ Ztschr. f. Immunitätsforsch., 1911, 10, p. 362.

²² Jour. Exper. Med., 1910, 12, p. 586.

²³ Ztschr. f. Immunitätsforsch., 1911, 8, p. 589.

²⁴ Arch. f. Exper. Path. u. Pharm., 1911, 65, p. 337.

²⁵ Arch. Internat. de Physiol., 1910, 10, p. 37.

²⁶ München. med. Wchnschr., 1911, 58, p. 670.

²⁷ Ztschr. f. Immunitätsforsch., 1911, 8, p. 740.

²⁸ Ztschr. f. Exper. Path. u. Ther., 1912, 10, p. 412.

first injection produced no marked changes. In sensitized animals, following a second injection, there was a marked leukopenia depending on the severity of the anaphylactic reaction. They report similar blood changes following the first injection of large doses of Witte peptone. Silk peptone had no appreciable effect. In their experiments they used as a sensitizing dose 20 cc of egg-white, and repeated this dose at varying intervals. They do not describe the symptoms accepted as anaphylactic, but apparently any animal becoming acutely ill was considered anaphylactic. They also obtained a leukopenia after a third injection of egg white without anaphylaxis resulting, so that leukopenia is not specific for the anaphylactic state.

Salus²⁹ sensitized guinea-pigs with 0.01 cc of horse serum and found that a nontoxic dose of Witte peptone (1-1.5 cc of a 10% solution) produced no effect. The same animals, however, died of acute anaphylactic shock after receiving an injection of 0.25 cc of horse serum. The horse serum did not sensitize to peptone, nor did an injection of peptone render the animal refractory to a second injection of horse serum. Similar results are reported by Besredka, Ströbel and Jupille.³⁰ Peptone shock, using Witte peptone, did not in the least protect guinea-pigs sensitized with horse serum against a second injection of horse serum. They also are of the opinion that the mechanism by which the symptoms are produced in "peptone intoxication" is entirely different from that concerned in anaphylactic shock.

Ritz³¹ observed that the injection of hypertonic (10%) salt solution into guinea-pigs sensitized with horse serum resulted in milder symptoms of anaphylaxis after the second injection of horse serum. Guinea-pigs received from 1.5-2.2 cc of a 10% Witte peptone solution which was followed by 0.9-1 cc of 30% sodium chlorid solution. The protective effect was not so marked nor as constant as in the case of true anaphylaxis. This author believes that his results furnish additional evidence of a close relationship between anaphylaxis and "peptone intoxication."

Kumagai and Odaira³² were not able to produce a specific anti-anaphylaxis by the use of Witte peptone. Guinea-pigs were sensitized with sheep serum, and after an incubation period the intoxicating dose was determined. By injecting sub-lethal doses of Witte peptone into sensitized animals they were able to inject twice the usual intoxicating dose of serum without killing the animal. Three times this dose was fatal. Animals which had recovered both from the injection of peptone and from a second injection of sheep serum were given a toxic dose of peptone. The animals showed only a slight protection against "peptone intoxication." The injection of peptone leads only to a slight nonspecific resistance to anaphylaxis, and from the results of their experiments the authors believe that peptone intoxication and anaphylaxis must be considered as separate and distinct phenomena.

In the laboratory of Schittenhelm and Weichardt,³³ an assistant developed severe respiratory symptoms from Witte peptone, and gave a local reaction when it was spread on the skin of his hand. Silk peptone had no such effect. This reaction might easily be accounted for by the presence of a minute amount of histamine.

²⁹ Biochem. Ztschr., 1914, 65, p. 381.

³⁰ Ann. de l'Inst. Pasteur, 1913, 27, p. 185.

³¹ Ztschr. f. Immunitätsforsch., 1912, 12, p. 644.

³² Ibid., 1912, 14, p. 391.

³³ Deutsch. med. Wehnschr., 1911, 37, p. 876.

(b) *Anaphylaxis with Products of Protein Digestion*.—Rosenau and Anderson³⁴ found that guinea-pigs sensitized with a mixture of toxin and antitoxic horse serum died when injected 27 days later with antitoxic horse serum to which various ferments had been added and allowed to stand over night at 15 C. The ferments used were takadiastase, pancreatin, rennin, mycrosin, invertin, emulsin, pepsin in acid solution, pepsin in alkaline solution, ingluvin, malt and papain. No attempt was made to determine to what extent, if at all, the proteins had been affected by enzyme action.

Realizing that the discrepancy in results obtained by investigations with Witte peptone might be due to the fact that it is such a heterogeneous mixture of substances, other workers have attempted to study the effects of proteolysis on the anaphylactogenic properties of proteins by using products prepared in the laboratory and purified as nearly as was possible by the use of existing chemical methods.

In his studies of the chemistry of anaphylaxis, Wells³⁵ found that tryptic digestion of bovine serum until but 8% of its nitrogen remained in coagulable form greatly reduced its sensitizing power. Such a serum sensitized guinea-pigs to normal bovine serum in doses of 0.004 cc but not in doses of 0.0004 cc. Normal bovine serum sensitized to itself in doses of 0.00001 cc. Sensitized guinea-pigs received 5 cc of the digestion mixture intraperitoneally without developing symptoms, showing that it had little or no intoxicating properties. Its effect on animals sensitized to bovine serum was the same. Digestion of serum to this point did not destroy its specificity, in that guinea-pigs sensitized to the digestion mixture did not react to horse serum or milk and were rendered refractory to these substances. After digesting for over 16 months³⁶ the mixture still contained traces of coagulable material (admixed with the trypsin which had been added?), but gave no biuret reaction. Guinea-pigs receiving doses of 1-5 cc were sensitized so that they reacted slightly but typically, to bovine serum injected 3 weeks later, the most marked reaction occurring in the pigs that had received the 5 cc doses; in no case was the reaction at all severe.

Similar experiments showed that peptic digestion of egg-white destroys its power to intoxicate sensitized guinea-pigs only when practically all coagulable protein has been destroyed. Egg albumin which had been acted on for 26 days by pepsin-HCl until no more coagulable protein was recognizable on heating still was able to sensitize guinea-pigs so that a subsequent injection of egg albumin produced moderate symptoms of anaphylaxis.

Albumoses, peptones, crystallizable amino-acids, etc., obtained by digesting egg white with pepsin and trypsin possessed no power to sensitize or intoxicate guinea-pigs. Some of the products of hydrolysis of coagulated egg albumin possessed a slight power of sensitizing to egg albumin. These experiments indicate that proteins cannot be decomposed much, if any, beyond the coagulable form without losing their anaphylactogenic properties.

Pick and Yamanuchi³⁷ found that beef serum digested with pepsin-HCl for 15 minutes was still able to sensitize to itself and to undigested beef serum. Rabbits sensitized with the digested serum showed symptoms of anaphylaxis when injected 6 days later with the same mixture and 9 days later when injected with normal serum. The sensitizing and intoxicating

³⁴ Hyg. Lab., Bull. 36, U. S. Pub. Health Serv., 1907.

³⁵ Jour. Infect. Dis., 1908, 5, p. 449.

³⁶ Ibid., 1909, 6, p. 506.

³⁷ Ztschr. f. Immunitätsforsch., 1909, 1, p. 676.

doses in young rabbits for native serum were not given so that a comparison between the anaphylactogenic properties of the original serum and the digested serum cannot be made. The dosage employed, however, is large so that apparently both the sensitizing and intoxicating properties of beef serum digested with pepsin-HCl for 15 minutes are low. A trypsin-digested mixture, free from coagulable protein and proteoses, yielded results which were not constant. The mixture was not able to sensitize to itself, but in one of a series of animals did sensitize to beef serum. They conclude from their experiments that pure native proteins yield most constant results in anaphylaxis. As the protein content diminishes, fewer positive results are obtained, and the sensitizing and intoxicating doses greatly increased, while the results become less constant.

In their studies of the antigenic properties of the split-products of casein, Gay and Robertson³⁸ found that a guinea-pig sensitized with 1 cc of 3% casein showed marked symptoms of anaphylactic shock when injected after 23 days with 5 cc of paranuclein. Paranuclein is a product of the partial digestion of casein by pepsin. In a similar manner paranuclein was found to sensitize animals to milk, and to a second injection of paranuclein itself.

A mixture of casein which had been digested with pepsin for 10 days at 36 C., did not sensitize either to a subsequent injection of the same mixture, or to a second injection of paranuclein.

Jobling and Strouse³⁹ obtained primary and secondary proteoses from Witte peptone by removing all coagulable material and then precipitating with one-half and full saturation of ammonium sulphate. Both fractions were toxic for guinea-pigs and produced death with necropsy findings similar to those in anaphylaxis. Egg white and casein were digested with leukoprotease and the proteoses obtained in the same way, yielded similar results.

Working with proteoses obtained by peptic digestion of beef fibrin prepared by the method of Adler (heteroalbumose and protoalbumose), of Haslam (heteroalbumose, protoalbumose alpha and beta, deutoalbumose alpha and beta), of Pick (heteroalbumose, protoalbumose, synalbumose and thioalbumose), pepsinfibrinpeptone-beta of Siegfried, and a mixture of abiuret products obtained by the digestion of fibrin with pepsin-trypsin-erepsin, Zunz⁴⁰ found that the heteroalbumoses and protoalbumoses were able to sensitize and intoxicate guinea-pigs and rabbits, while synalbumose sensitized only. In animals treated with heteroalbumose, protoalbumose or synalbumose the symptoms of anaphylactic intoxication are not generally so marked as in serum sensitized animals. They usually appear after a definite latent period and are not always marked. In these cases it was necessary to use the lowering of rectal temperature as a criterion of anaphylaxis in guinea-pigs.

If the serum of an animal injected with heteroalbumose or protoalbumose be withdrawn after a suitable interval and incubated with the proteose used for sensitization, a solution is obtained which produces the symptoms of anaphylactic intoxication when injected into a normal animal.

The results of Zunz' experiments clearly show that proteoses are not as effective as serum in the production of anaphylaxis, much larger doses being required to produce less marked symptoms. Specificity is not marked, since an animal sensitized with one proteose will react with any of the other anaphylactogenic proteoses and with beef serum. Species specificity, how-

³⁸ Jour. Exper. Med., 1912, 16, p. 470.

³⁹ Ibid., 1913, 18, p. 591.

⁴⁰ Ztschr. f. Immunitätsforsch., 1913, 16, p. 580.

ever, is shown, inasmuch as an animal sensitized with proteoses obtained from beef fibrin does not react with horse serum.

Friedberger and Joachimoglu⁴¹ sought to repeat Zunz' work with heteroalbumoses and protoalbumoses. They used preparations furnished them by Zunz. On examining the protocols in the latter's report, they were surprised at the large dose of beef serum used, a dose which in their experience proved toxic to normal animals; 0.15-0.20 c.c. per 100 gm. body weight was lethal for young guinea-pigs. The lethal dose for animals injected 11 days previously with heteroalbumose or protoalbumose was the same. Such animals showed no symptoms when injected with large doses of horse serum. They therefore attribute the greater susceptibility of guinea-pigs to beef serum as compared with horse serum to the difference in toxicity of the two serums, and not to true sensitization. As compared with normal controls, pigs previously treated with albumose do not show an increased susceptibility to beef serum.

Zunz and György⁴² then repeated some of the experiments with proteoses, and again reported that guinea-pigs sensitized with either hetero- or protoalbumose gave anaphylaxis reactions when injected with either or with ox serum, but not with horse serum. The question of the primary toxicity of beef serum as a possible explanation of these results was not discussed.

The sensitizing power of heteroalbumoses seemed to be more marked than their ability to produce anaphylaxis in animals treated with these proteoses. Thus, in such animals the intravenous injection of hetero- or protoalbumose resulted only in mild symptoms of intoxication or even no symptoms whatever. They suggest that perhaps there are varying grades in the sensitizing ability of two heteroalbumose or protoalbumose preparations obtained in exactly the same manner.

A large series of experiments with peptic, tryptic and acid hydrolytic cleavage products of beef and hog muscle is reported by Hailer.⁴³ In addition, a number of commercial peptone and meat extract preparations were used. The products were tested for coagulable protein, biuret reaction, nitrogen content and precipitability by a homologous precipitin serum. From the results obtained Hailer concludes that it is undoubtedly possible to sensitize guinea-pigs with completely digested protein mixtures (free from coagulable protein, and broken down to the final building stones—the amino-acids—by boiling with sulphuric acid); but that this sensitization is by no means specific since anaphylactic symptoms develop after reinjection of totally unrelated proteins. Despite intensive treatment with a particular kind of protein specific sensitization did not occur when the solution contained large quantities of cleavage products. Similar results were obtained with the commercial products except that reactions were more marked when native protein was present in addition to the cleavage products.

These results are not in agreement with those obtained by Wells³⁶ who found that tryptic digested serum containing but 8% coagulable nitrogen sensitized to beef serum (40 times the dose of native beef serum required) but did not sensitize to horse serum or milk and were not rendered refractory to these substances. Hailer's conclusions appear to be based on insufficient experimental evidence. Thus, he says that relatively toxic doses were selected because it seemed necessary to flood the animal with large quantities of

⁴¹ *Ibid.*, 1914, 22, p. 522.

⁴² *Ibid.*, 1914, 23, 296.

⁴³ *Arb. a. d. k. Gsndhtsamte*, 1914, p. 527.

native protein when using substances of relatively weak sensitizing power. As much as 0.6 cc of beef serum was injected intracardially. Friedberger and Joachimoglu⁴¹ found that the lethal dose of beef serum for normal guinea-pigs was 0.15-0.20 gm. per 100 gm. body weight, and while Hailer inactivated his serum, definite symptoms might have resulted from the injection of such doses of beef serum. Since none of his animals died, this remains as a possibility. Suitable controls were not used, as the author depended on the toxic doses of human, horse and swine serums as determined by Uhlenhuth and Händel. No attempt was made to test the intoxicating properties of the digestion mixtures or commercial preparations. The statement that the presence of native protein interferes with the specificity of anaphylaxis by digestion mixtures is contrary to the experience of Wells, whose findings in this respect have already been quoted.

In a series of experiments reported by Schmidt,⁴² deutoalbumose obtained from Witte peptone according to the method of Kutcher was unable to sensitize or intoxicate a sensitized animal.

(c) *Vaughan's Protein Poison*.—The work of Vaughan and his students also represents an attempt to prove that anaphylaxis is the result of parenteral digestion of proteins. His theory of anaphylaxis as recently stated in a publication from his laboratory, is that the parenteral introduction of a protein into an animal, the guinea-pig for example, leads to the production of a specific proteolytic ferment by the cells of that animal. Following a suitable incubation period, the injection of a second dose of this same protein, parenterally, results in the liberation of 'the specific enzyme which then digests the protein molecule with the immediate production of large quantities of poison, sufficient under proper conditions, to kill the animal (Pryer⁴³). The experiments of Vaughan on the "protein poison" have served as the basis of this theory.

By extracting egg-white with boiling alcohol (78 C.) containing 2% sodium hydroxid, Vaughan and Wheeler⁴⁴ were able to split the protein into a poisonous and nonpoisonous fraction. Earlier experiments (1903) had shown that colon bacillus protein could be thus broken up, and subsequent work in Vaughan's laboratory⁴⁵ has shown that all proteins, animal, bacterial and vegetable may be broken up in this manner. When injected into animals, the toxic fraction produces symptoms identical in every particular with those following a second injection of egg white into an animal sensitized against egg white. The minimum lethal dose of the purest preparation of protein poison thus far isolated is 0.0005 gm. (Pryer). Wells⁴⁶ found the minimal lethal dose of egg white injected into the circulation of sensitized guinea-pigs to be $\frac{1}{10}$ to $\frac{1}{20}$ mg.

Chemical examination of the poisonous product indicated only that the carbohydrate group was absent. For a time Vaughan considered it best to look on the poisonous fraction merely as a cleavage product of whole protein, but still a protein. Recently, however, he has expressed the opinion that "the so-called peptone poison, proteoses and the protein poison are closely related bodies" (Vaughan⁴⁷).

⁴⁴ Univ. of Cal. Pub. in Pathology, 1916, 2, p. 157.

⁴⁵ Jour. Lab. and Clin. Med., 1916, 1, p. 490.

⁴⁶ Jour. Infect. Dis., 1907, 4, p. 476.

⁴⁷ Jour. Lab. and Clin. Med., 1916, 1, p. 400.

⁴⁸ Jour. Infect. Dis., 1908, 5, p. 449.

⁴⁹ Jour. Am. Med. Assn., 1916, 67, p. 1559.

Armit⁵⁰ was unable to confirm Vaughan's original experiments. He found that when pure crystallized egg albumin is employed the portion corresponding to the haptophore fraction is unable to sensitize normal guinea-pigs to itself, to the other fraction, or to whole egg albumin. On the other hand, the toxophore fraction is capable of sensitizing to a slight extent. Under certain circumstances it is able to intoxicate when used for a third injection. The hypersusceptibility to pure albumin was not interfered with by the injection of either fraction. On subsequent testing with egg white the guinea-pigs were found to be hypersensitive.

Based on a wide experience in the study of proteoses and "peptone intoxication," Underhill and Hendrix⁵¹ report that Vaughan's crude soluble poison from casein has a more marked physiologic action than any proteose with which they have worked. Its action on blood pressure and blood clotting closely resembles that of the proteoses. Vaughan's preparation was found to be toxic for rabbits in relatively small doses, differing in this respect from the proteoses. By boiling with dilute HCl the toxicity of the protein poison was destroyed, indicating that it is a toxic product of protein hydrolysis.

(d) *Precipitins with Derived Proteins*.—In his immunological studies of eel serum, Tchistovitch⁵² attempted to produce precipitins against peptone (presumably Witte's). After 5 or 6 injections of 5 cc each of 10% peptone solution into a rabbit, no evidence of a precipitin against peptone could be obtained. The first positive results with the use of Witte peptone as antigen were those of Myers.⁵³ The commercial product was dissolved in salt solution and the coagulable protein removed by boiling. The cooled filtered solution (concentration not mentioned) was injected intraperitoneally into rabbits. The details of the experiments are not given. Immunization led to the appearance of substances in the serum which produced a precipitum in Witte peptone at 37 C. Control experiments gave absolutely negative results. Heating at 56 C. for half an hour weakened the precipitating power of the serum, but the addition of normal serum restored the original strength. Peptone solution to which normal rabbit serum was added remained perfectly clear. Since the details of these experiments have not been published, a critical discussion is not possible. Later work has not confirmed the results with Witte peptone. Heat inactivation and serum reactivation is not a phenomenon generally observed with precipitins, and this seems to be the only recorded instance in which it occurred.

The statement of Myers that the precipitate formed by the reaction of Witte peptone with a serum immunized against it did not give a biuret reaction, was investigated by Bashford⁵⁴ who was able to immunize two goats against Witte peptone, and obtained a large quantity of precipitate by treating the serum with Witte peptone. Comparison of analyses of the precipitated product and the mother substance did not show any striking difference in composition. Bashford points out that proteoses, in the presence of other protein substances in neutral solutions, may yield precipitates which are not the product of a specific immune reaction.

Obermayer and Pick⁵⁵ worked with tryptic digested mixtures of pure proteins that did not show the presence of unaltered protein. The mother sub-

⁵⁰ Ztschr. f. Immunitätsforsch, 1910, 6, p. 703.

⁵¹ Jour. Biol. Chem., 1915, 22, p. 465.

⁵² Ann. de l'Inst. Pasteur, 1899, 13, 406.

⁵³ Lancet, 1900, 2, p. 98; Cent. f. Bakteriöl., 1900, 28, p. 237.

⁵⁴ Quoted by Nuttall, Blood Immunity and Relationship, Appendix, Note 2.

⁵⁵ Wien, klin. Rund., 1902, 16, p. 277.

stances were egg white, a globulin, conalbumin—the noncrystallizable portion of egg white, and ovomucoid. The injection of trypsin digestion products of these substances into animals led to the rapid appearance of immune products. They believe, therefore, that precipitinogen is not complete protein. The action of pepsin-HCl on proteins which readily produce immune bodies is to destroy their antigenic properties, even while there are considerable quantities of albumoses and peptones present in solution. Witte peptone in their experience had no antigenic properties. It is impossible to give a critical review of this paper by Obermayer and Pick because they give only the results of their experiments and omit a detailed description of the digestion mixtures, the methods used in immunization and the number of experiments on which their results were based. A second paper by the same authors⁵⁶ consists of a discussion of the biochemistry of the precipitin reaction with experiments designed to determine on what chemical group in the protein molecule species specificity depends. A biuret-free preparation obtained by long continued autolysis of beef pancreas appeared totally inactive in one experiment. On the other hand, if coagulated beef serum or egg white be subjected to the action of trypsin an immune serum can be obtained with the products of digestion even after the biuret reaction has disappeared. Such an immune serum has a very narrow range of reaction in that it precipitates only the digestion mixture. Its species specificity remains intact, since it does not react with the products of tryptic digestion of horse serum. Immunization of rabbits with products of oxidation of proteins by potassium permanganate in alkaline solution led to the formation of precipitins which were strongly species specific.

After many attempts Michaelis and Oppenheimer⁵⁷ were unable to obtain a precipitin by the use of peptic digestion products of proteins which would precipitate the mother substance. They used Riedel peptone, which is obtained through peptic digestion of beef fibrin, Merck egg peptone (peptic digested egg white precipitated with alcohol and dried in vacuo) and pure deutero-albumose obtained from beef by acid hydrolysis. Both commercial preparations probably consisted of mixtures of albumoses. Fifteen animals were used in their experiments. In no instance did the serum of these animals show a specific precipitin. The ability to form a precipitate with a specific immune serum was lost even when considerable heat coagulable material was present in the digestion mixture.

In the case of tryptic digestion, as long as coagulable protein was demonstrable in the solution, it was precipitable by precipitin. After digestion for several weeks with large quantities of trypsin, until protein had entirely disappeared, the precipitability of the mixture by precipitin was completely lost, and it was no longer possible to obtain a precipitin for the mother protein by injecting this substance.

Rostoski and Sacchonagi⁵⁸ worked with pepsin and trypsin digestion products of horse serum albumin. They were not able to get rid of the last traces of coagulable protein in the tryptic digestion. Albumoses were obtained by half and full saturation with ammonium sulphate. The filtrates contained peptones. The serum of injected animals yielded a definite precipitate when added to the solutions used for injection. Very marked results were obtained with the ring test of Ascoli, in which serum and solution were placed in narrow

⁵⁶ Wien. klin. Wchnschr., 1906, 19, p. 327.

⁵⁷ Arch. f. Physiol., 1902, Supplement, p. 343.

⁵⁸ Ztschr. f. klin. Med., 1903-04, 51, p. 187.

test tubes. After several minutes a heavy ring-shaped precipitate formed, almost diffuse, finally falling to the bottom. Numerous control experiments using normal serum against the various substances were always negative. The precipitins in this case were not specific, in that the serum of an animal immunized against one of the solutions reacted with all of them. Rostoski and Sacchonagi conclude from their experiments that precipitins can be produced against the products of gastric and pancreatic digestion, even the peptones. They ascribe the failure of previous workers to the use of related proteins.

Michaelis⁵⁹ injected a preparation of beef serum partially digested with pepsin-HCl, intraperitoneally into a rabbit at 4-day intervals. Six days after the third injection the serum of the animal was tested. It showed an active precipitin for the digested beef serum producing a marked opalescence almost as soon as the mixture was added. This rapidly turned to a precipitate and settled out. The reaction did not occur in as high dilution as native serum precipitin. With small amounts of normal horse serum a precipitate was formed which dissolved on the addition of an excess of serum. When tested with pseudoglobulin there was no reaction; it reacted well, however, with euglobulin and albumin. If enough serum was added to redissolve the precipitate first formed, the addition of a drop of digestion mixture produced an opalescence. The precipitin against partially digested serum did not react with a digestion mixture from which all coagulable material had been removed either by prolonged digestion or by heating.

Pozerski and Pozerska⁶⁰ were unable to demonstrate the presence of specific precipitins in the serum of a dog immunized against Witte "peptone."

Levene⁶¹ worked with proto- and deutoalbumoses obtained from Witte "peptone" by precipitation with one-half and full saturation with ammonium sulphate. The serum of rabbits injected with either proteose formed a precipitate with both antigens. Relatively large quantities of serum (as much as 0.4 cc) and antigen (2% solution) were used in the tests. Schmidt⁶² used deutoalbumose from Witte "peptone" and did not obtain a precipitin reaction with the antiserum.

Recently Lampé⁶³ has reported positive results with various peptones. These were obtained from the crystalline lens, brain, placenta, thyroid, thymus, lung, silk and gliadin. The smallest quantities used activated hemolysis. Normal serum was found to contain no antibodies to these peptones, but antibodies to some may occur in human serum in disease or in rabbits after immunization. Specificity was not absolute in that following protein injections antibodies to the corresponding peptones appeared. Unfortunately I have been unable to gain access to the original article and am thus unable to determine just what tests for antibody production were employed, but the ability of a substance to activate hemolysis is not a sufficient test of its antigenic properties.

(c) *Complement Fixation with Protein Derived Products.*—Friedberger and Gay and Robertson using the substances mentioned under the discussion of the anaphylaxis reaction were unable to demonstrate the presence of an antibody to any of the substances except whole protein. Schmidt also reports negative results with his deutoalbumose.

⁵⁹ Deutsch. med. Wchnschr., 1902, 28, p. 733.

⁶⁰ Compt. Rend. Soc. Biol., 1911, 70, p. 592.

⁶¹ Jour. Med. Research, 1904, 12, p. 195.

⁶² Univ. of Cal. Pub. in Pathology, 1916, 2, p. 157.

⁶³ Deutsch. Arch. f. klin. Med., 1916, 119, p. 113. Physiological Abstracts, 1916, 1, p. 224.

By digesting proteins (horse serum) with an alcoholic solution of sulphuric acid (48 hours at 62-63 C.), Landsteiner and Prasek⁶⁴ were able to produce an immune serum in rabbits which reacted with similarly modified serum proteins of various animals (beef, chicken, rabbit) and even with edestin but not with unaltered horse serum.

Landsteiner and Jablons⁶⁵ report that rabbit serum treated with alcoholic sulphuric acid produces complement binding antibodies when injected into rabbits.

SUMMARY OF LITERATURE

The bulk of evidence indicates that "peptone shock" is not an anaphylactic phenomenon. Other substances totally unrelated to anaphylactogens, such as saponins and hirudin may give similar symptoms. The work of Underhill and others has shown quite conclusively that the proteoses contained in the various "peptone" preparations are responsible for their physiologic action. "Peptone shock" differs fundamentally from anaphylaxis in the absence of the phenomenon of sensitization following a suitable incubation period, and in the relatively large quantity of "peptone" required to produce a reaction. Experiments with products of protein digestion have shown that proteins cannot be disintegrated much if any beyond the coagulable form without losing their sensitizing and intoxicating properties. Positive experiments reported with proteoses as anaphylactogens have not been fully confirmed.

The single recorded instance of precipitin formation against Witte peptone has never been confirmed, all subsequent work showing that it has no antigenic properties.

Most of the precipitin experiments with protein digestion products were undertaken to determine on what group in the protein molecule specificity depends, rather than to test the effect of disintegration on their antigenic properties. The entire literature on precipitins is in a chaotic state, the great bulk of it consisting of theoretical discussions. Instead of testing against the digestion mixtures, the serums obtained by their use have often been tested against the mother substance alone. Separating the wheat from the chaff, what little remains seems to parallel the results with the anaphylaxis reaction. Precipitinogen seems to be more resistant to tryptic digestion than to peptic digestion, corresponding to proteins in general in this respect.

Experiments with the complement fixation reaction have been negative.

⁶⁴ Ztschr. f. Immunitätsforsch., 1913, 20, p. 211.

⁶⁵ Ibid., 1914, 20, p. 618.

We might mention at this point that the evidence for the proteose nature of toxins is doubtful, and that Whipple^{65a} considers intestinal intoxication due to proteoses.

THE CHEMISTRY OF PROTEOSES

The proteoses are not a chemically definable group of substances, inasmuch as their exact chemical composition is practically unknown. Our knowledge of proteoses has resulted largely from attempts to determine the structure and composition of the protein molecule through methods of analysis. By hydrolytic cleavage, proteins are decomposed, yielding products of lower molecular weight. Among the first of these are the proteoses regardless of the agent employed for hydrolysis, whether by the action of enzymes, dilute acids or superheated steam. So that proteoses, or albumoses, as they are often called have been defined as a group of derived proteins. Analysis has shown that they differ but little in their fundamental composition from the mother proteins.

Albumoses are identified almost wholly by their physical properties and physiologic action. That they consist of smaller molecules than the proteins from which they are derived is evidenced by the fact that they are somewhat more diffusible. Their separation and classification have been largely worked out by Kühne and his pupils, and is based on the fact that they are precipitated by solutions of neutral salts of different strengths.

Kühne and Chittenden⁶⁶ divide the primary albumoses into proto- and hetero-albumose. During peptic digestion these give rise to the secondary albumoses or deuto-albumoses. Differences in solubility and precipitability by sodium chlorid led to the separation of four different albumoses. Kühne's⁶⁷ method of separating albumoses from peptones consists in saturating with ammonium sulphate while hot, a solution containing a mixture of the two of neutral, alkaline and acid reactions. Although apparently simple, their method does not always succeed, and Kühne and Chittenden have found that the filtrates which are supposed to contain only peptones sometimes contain definite quantities of proteoses. The difficulty seems to be in the adjustment of the reaction at the different stages of precipitation.

Proteoses thus obtained were found to be water-soluble; with sodium chlorid and nitric acid they formed precipitates in the cold which dissolved on heating, and they all gave the biuret reaction.

The work of Kühne and Chittenden was carried a step farther by Neumeister⁶⁸ who perfected a method of separating deutoalbumoses from a

^{65a} Jour. Am. Med. Assn., 1916, 67, p. 15.

⁶⁶ Ztschr. f. Biol., 1884, 20, p. 11.

⁶⁷ Ibid., 1892, 29, p. 1.

⁶⁸ Ibid., 1887, 23, p. 381.

mixture of albumoses. Neumeister's method is based on the conception that, in the process of protein cleavage, albumoses are formed in two stages. The first stage results in the formation of proto- and hetero-albumoses, which he has termed primary albumoses; in the second stage each of the primary albumoses yields a deutoalbumose, or secondary albumose.

E. P. Pick⁶⁹ working with Witte peptone was able to isolate four fractions by precipitation with ammonium sulphate of varying degrees of saturation.

The method of fractional precipitation of proteoses has been severely criticized by Haslam⁷⁰ who called attention to the fact that there are no chemical tests by which we are able to prove that the various fractions are not mixtures, and that reprecipitation does not serve to remove all traces of impurities, especially other albumoses. In order to avoid this difficulty, Haslam suggests that where possible, the filtrate should be tested for the substance which it is desired to remove by precipitation. Where no such test exists, the amount of organic nitrogen should be determined by the Kjeldahl method in the original filtrate after resolution and reprecipitation. The substance should not be considered pure, until the organic nitrogen in the filtrate becomes constant. Washing the precipitate was found to be of no value because the impurities are intimately admixed with the albumoses and washing affects only the surface particles.

PRELIMINARY EXPERIMENTS WITH WITTE "PEPTONE" PRODUCTS

It seemed desirable to test the antigenic properties of Witte "peptone," in the first place to get an idea of the activity of proteoses in this respect, and in the second place to determine if possible why the results of other workers are so conflicting. Three preparations were obtained as follows:

1. Hot alcohol soluble proteose prepared according to the method of Gibson⁷¹ who proved by its physiologic activity on dogs that it was a true proteose. Witte "peptone," 150 gm., were boiled several hours with 1 liter of 80% alcohol under a reflux condenser. The alcoholic solution was filtered through a hot water funnel and the proteose precipitated in a semi-crystalline form in a freezing mixture. The extraction was repeated several times and the fractions combined. The final product was pulverized in a mortar, yielding a brownish-white powder readily soluble in water. From a watery solution it could be precipitated by $\frac{2}{3}$ saturation with ammonium sulphate and the filtrate gave only an opalescence with an excess of ammonium sulphate. The filtrate from $\frac{3}{4}$ saturation gave no further precipitate on the addition of saturated ammonium sulphate. This preparation was used as antigen 1.

2. Cold alcohol soluble material prepared by evaporating to dryness the filtrate from the above and pulverizing in a mortar. This was a light yellow powder, readily soluble in water, and was used as antigen 2.

3. The residue from the alcohol extraction was dissolved as much as possible in hot water, evaporated to a syrupy consistence, and precipitated with 3 times its volume of 95% alcohol, yielding a sticky mass. This was washed with absolute alcohol, followed by ether and dried in a desiccator. The dried material was powdered as above and used as antigen 3.

4. Beef serum was used as control antigen 4.

⁶⁹ Ztschr. f. Physiol. Chem., 1898, 24, p. 246.

⁷⁰ Jour. Physiol., 1905, 32, p. 267; 1907, 36, p. 164.

⁷¹ Philippine Jour. Science, 1914, 9B, p. 499.

ANIMAL EXPERIMENTS

The first series of animals were immunized by the intensive method. Intraperitoneal injections were given on three successive days of 0.5, 1.0 and 1.5 gm., respectively, and the animals bled on the 10th day after the first injection. The serums were then tested for precipitins and complement binding substances. For the precipitin reaction a 1% antigen solution was used in dilutions varying from 0.5-0.0009 c c with the usual controls. In testing out each serum all the antigens were used to determine nonspecific reactions. For the complement fixation reactions a 1% solution of the antigens were used in dilutions ranging from $\frac{1}{20}$ to $\frac{1}{10,240}$ c c with suitable controls. In this case also all the antigens were tested against each serum. The serums of animals injected with antigens 1, 2 and 3 failed to show the presence of antibodies for any of the antigens used. Animals treated in the same way, using 5, 10 and 15 c c of beef serum showed in the case of precipitins a positive reaction down to the 5th tube, and complete inhibition in the first 4 tubes with partial inhibition in the rest. With the other preparations as antigens, using the same system, there were no reactions.

In order to be certain that the negative results were not due to insufficient treatment of animals, another series of rabbits were injected with increasing doses of antigen at 3-day intervals, beginning with 0.5 gm., and increasing by 0.1 gm. at each subsequent injection. These animals were injected over a period of one month. The results were the same, and no evidence of antibody formation could be demonstrated. Therefore, the three fractions prepared from Witte's "peptone" possessed no power to stimulate the production of precipitins or complement binding antibodies, either for themselves or for beef serum — the mother substance. Neither did they react with the serum of rabbits immunized against beef serum.

Anaphylaxis experiments showed that the Witte "peptone" antigens possessed only very slight anaphylactogenic properties. Guinea-pigs received intraperitoneal injections of 25 mg. of the substance to be tested and 3 weeks later a second injection of 50 mg. In only one or two cases did the animals become sick. In no case did such severe reactions result as follow on the re-injection of beef serum into an animal sensitized to beef serum. For the most part the reactions consisted of vigorous scratching 15 minutes after reinjection. Guinea-pigs sensitized with Witte "peptone" products did not react to beef

serum, nor was the reverse combination any more effective. Preliminary experiments with normal guinea-pigs showed that the preparations in the dosage employed were nontoxic. The protocols are contained in table 1.

TABLE 1
ANAPHYLAXIS EXPERIMENTS WITH PRODUCTS FROM WITTE'S "PEPTONE"

Sensitizing Dose (Intraperitoneally)	Days Interval	Second Injection	Results
1. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	Slight roughing, scratching
2. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	Scratching
3. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	Scratching
4. 0.025 gm. Antigen 1	19	0.050 gm. Antigen 1	No symptoms
5. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	No symptoms
6. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	No symptoms
7. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	Scratching
8. 0.025 gm. Antigen 2	19	0.050 gm. Antigen 2	No symptoms
9. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Roughened hair, scratching
10. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Scratching
11. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Slightly upset, rapid resp.
12. 0.025 gm. Antigen 3	19	0.050 gm. Antigen 3	Scratching
13. 0.025 gm. Antigen 1	19	0.1 c c heated beef serum	No reaction
14. 0.025 gm. Antigen 2	19	0.1 c c heated beef serum	Scratching
15. 0.025 gm. Antigen 3	19	0.1 c c heated beef serum	No reaction
16. 0.1 c c heated beef serum	21	0.1 c c heated beef serum	Typical shock, recovery
17. 0.1 c c heated beef serum	21	0.050 gm. Antigen 1	No reaction
18. 0.1 c c heated beef serum	21	0.050 gm. Antigen 2	No reaction
19. 0.1 c c heated beef serum	21	0.050 gm. Antigen 3	Scratching, late paralysis, not specific
20. 0.1 c c heated beef serum	21	0.050 gm. Antigen 3	No reaction
21. 0.1 c c heated beef serum	21	0.050 gm. Antigen 3	No reaction

Beef serum does not sensitize to products of Witte "peptone," nor does it produce "shock" in animals sensitized to such products.

Witte "peptone" preparations, therefore, possess a slight power of sensitizing to themselves. They are not able to sensitize to beef serum, nor do they produce symptoms of anaphylactic intoxication in guinea-pigs sensitized to beef serum.

EXPERIMENTS WITH PROTEOSES FROM EGG WHITE

(a) *Method of Preparation.*—In order to avoid the possibility of introducing protein substances which might be difficult to get rid of in a digestion mixture, enzymes were not used. The method employed was essentially that of Chittenden, Mendel and Henderson.⁷² The whites of 6 dozen eggs were coagulated by pouring slowly into a large volume of boiling water to which enough acetic acid had been added to make it distinctly acid to litmus paper. The coagulum was collected on a clean towel, the water squeezed out as much as possible and ground through a fine-meshed copper sieve, in order to obtain the maximum surface for hydrolytic action. The coagulated egg white in a finely divided state, was suspended in an equal volume of water and autoclaved for 10 hours with steam under 10 lbs. pressure. The mixture was then filtered and the filtrate slightly acidified with acetic acid to precipitate any coagulable protein present, again filtered, and the filtrate rendered slightly alkaline with ammonium hydroxid. The neutralization precipitate was filtered

⁷² Am. Jour. Physiol., 1898-99, 12, p. 142.

off, and the solution boiled on the water-bath to drive off any excess ammonia, and finally concentrated to a small volume. This final product was filtered until clear. To the filtrate was then added enough cold saturated $(\text{NH}_4)_2\text{SO}_4$ solution to make it $1/4$ saturated, that is, to every 300 cc of filtrate 100 cc of saturated sulphate solution was added. The brownish sticky precipitate was filtered off and allowed to dry on the filter paper. In the same way products were obtained by adding to the filtrate enough saturated $(\text{NH}_4)_2\text{SO}_4$ solution to yield $1/3$, $1/2$, $2/3$, $3/4$ and full saturated fractions, that is, to the filtrate of the $1/4$ saturated fraction was added the calculated volume necessary to bring the concentration to $1/3$ saturation, etc. The insoluble residue from the original filtrate was again suspended in water and again autoclaved. When the yield in proteoses became small a final hydrolysis with 0.8% HCl was attempted and the residue brought into solution. In order to obtain enough material for immunizing animals it was necessary to repeat the process many times. The yield of proteoses was inconstant at different times, in some instances being abundant, while in others quite disappointing. This has been the experience of most workers with proteoses, and makes the task of obtaining proteoses by hydrolysis tedious and time-consuming. Obviously we are dealing here with a chemical reaction which cannot be controlled. At one time the process may be interrupted at a point when the maximum yield of proteoses results, at another time when the disintegration of the molecule has gone beyond this stage. In the case of a compound of the complex structure of the protein molecule there is no way of determining the stage of optimum production of proteoses. Where the conditions of chemical equilibrium are changing as constantly as in the hydrolysis of proteins, the time when the process should be interrupted is largely a matter of trial and error.

The preparations were purified by dissolving the dried material on the filter papers, discarding the insoluble portions, and determining the amount of ammonium sulphate present by the Folin aeration method.⁷³ For this purpose 10 cc of the solution to be tested was placed in the receiving cylinder of the Folin apparatus. This was then covered with 5 cc paraffin oil, and 5 gm. dry Na_2CO_3 dusted over the layer of oil. The cylinder was stoppered quickly, and by means of a current of air the liberated ammonia was passed into another cylinder containing 20 cc of $\text{N}/20 \text{ H}_2\text{SO}_4$. Vigorous aeration was continued for $1\frac{1}{2}$ hours, after which the acid solution was titrated with $\text{N}/10 \text{ NaOH}$ using congo red as indicator. From the amount of ammonia thus determined, the concentration of $(\text{NH}_4)_2\text{SO}_4$ in the total volume of solution was calculated and sufficient saturated $(\text{NH}_4)_2\text{SO}_4$ solution added to bring it to the desired concentration ($1/4$ saturation, $1/3$ saturation, etc., in the corresponding fractions). The filtrate was added to the succeeding fraction. The precipitates were washed with the corresponding saturation of $(\text{NH}_4)_2\text{SO}_4$ solution and the whole process repeated. The final products were obtained by precipitation with 3 volumes of alcohol, washing with a small amount of ether, and drying in a desiccator. The result in all cases was a white amorphous powder readily soluble in water. The yield was as follows: $1/4$ saturated fraction, 0.7 gm.; $1/3$ saturated fraction, 4.2 gm.; $1/2$ saturated fraction, 11 gm.; $2/3$ saturated fraction, 25 gm.; $3/4$ saturated fraction, 12 gm.; $4/4$ saturated fraction, 49 gm.

⁷³ Mathews, *Physiological Chemistry*, 1916, p. 961.

Sufficient material for testing the physiologic action of the preparations was obtained only with the $\frac{1}{2}$, $\frac{2}{3}$, $\frac{3}{4}$ and $\frac{4}{4}$ saturated products. These were injected intravenously into dogs in concentrations of 0.06 gm. per kg. of body weight and showed in all cases the typical drop in blood pressure characteristic of all proteoses.

The protocols of our immunization experiments show that the small amounts of ammonium sulphate contained in the preparations did not interfere with the biologic reactions.

(b) *Animal Experiments.*—Rabbits were used to test for the production of precipitins and complement binding antibodies. In the first series of experiments, rabbits were injected in pairs with each fraction as follows: 0.1 gm. dissolved in 5 c.c. of water was injected intravenously on each of 3 successive days, the animals allowed to rest 3 days, after which 3 injections of 0.1 gm. each were again given on successive days. Each animal thus received 0.6 gm. of the preparation to be tested. A second series of rabbits was injected intravenously with proteose preparations, a pair of animals for each fraction, starting with 0.1 gm. and increasing by 0.1 gm. at each succeeding injection, space at 3-day intervals over a period of 1 month. The rabbits were bled and their serums tested for antibodies during the course of treatment, and the final injection consisted of an intraperitoneal injection of 0.5 gm., the deciding test being made with serum withdrawn on the 10th day after the last injection. As controls, rabbits were treated with filtered fresh egg white solution, in exactly the same way using 0.1 c.c. egg white for each 0.1 gm. proteose. Owing to the small yield of proteoses of $\frac{1}{4}$ saturation with ammonium sulphate, only a limited number of experiments were possible with this fraction.

The technic in testing for precipitins and complement binding antibodies was the same as described in connection with the experiments on Witte "peptone" products. The serums of animals injected with $\frac{1}{4}$, $\frac{1}{3}$, $\frac{1}{2}$ and $\frac{2}{3}$ saturation products showed no evidence of production of either precipitins or complement binding antibodies.

The results of the experiments show that the $\frac{3}{4}$ and $\frac{4}{4}$ saturated products were not as efficient antibody producers as egg white, but that they possess definite antigenic power for both precipitin and complement binding antibodies. In the complement fixation experiments the possible anticomplementary effect of ammonium sulphate contained in the preparations was carefully controlled. In treating rabbits for antigen production we found that two of our animals died suddenly following the third intravenous injection, with typical symptoms of anaphylactic shock. Such reactions never occurred with any of the other fractions, and furnish additional evidence that the $\frac{3}{4}$ and $\frac{4}{4}$ saturated fractions possess antigenic properties not possessed by the others.

The following are typical protocols of experiments with 3/4 and 4/4 saturation products and egg white as control.

TABLE 2
COMPLEMENT FIXATION TESTS.* EGG-WHITE IMMUNE RABBIT SERUM

Antigen† Dilution 1% Solution	1/4 Saturated	1/3 Saturated	1/2 Saturated	2-3 Saturated	3/4 Saturated	4/4 Saturated	Egg- white
1. 1:20	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	++	++	++++
2. 1:40	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++++
3. 1:80	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++++
4. 1:160	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++++
5. 1:320	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++++
6. 1:640	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	+	Slight fixation	++
7. 1:1,280	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Slight fixation	Slight fixation	+
8. 1:2,560	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Slight fixation	Slight fixation	+
9. 1:5,120	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Slight fixation	Slight fixation	+
10. 1:10,240	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
11. Normal salt	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
12. 1:20 + nor- mal rabbit serum (56 C.)	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete
13. 1:20 + nor- mal salt	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete	Hemolysis complete

* The hemolytic system employed was: Amboceptor—rabbit serum immune to sheep's corpuscles, 2 units. Complement—fresh guinea-pig serum, titrated before use, 1.2 units. Sheep's corpuscles, thoroughly washed fresh, 2.5% suspension. Anticomplementary dose of antigen determined and 1/4 anticomplementary dose used.

† 0.1 cc antiserum (56 C. ½ hr.) in each of first 10 tubes, normal salt sol. q. s. 4 cc in all tubes.

TABLE 3
PRECIPITIN TEST. EGG-WHITE-IMMUNE RABBIT SERUM. 0.1 CC ANTISERUM IN EACH OF FIRST 10 TUBES, NORMAL SALT SOLUTION Q. S. 2 CC IN ALL TUBES

Dilution of Antigen 1% Solution C C	Antigens						Egg-white Saturated
	1/4 Saturated	1/3 Saturated	1/2 Saturated	2/3 Saturated	3/4 Saturated	4/4 Saturated	
1. 0.5	—	—	—	—	slight	slight	+++
2. 0.25	—	—	—	—	slight	slight	+++
3. 0.125	—	—	—	—	slight	slight	+++
4. 0.0625	—	—	—	—	slight	slight	+++
5. 0.03125	—	—	—	—	—	—	+
6. 0.0156	—	—	—	—	—	—	+
7. 0.0078	—	—	—	—	—	—	+
8. 0.0039	—	—	—	—	—	—	+
9. 0.0019	—	—	—	—	—	—	+
10. 0.0009	—	—	—	—	—	—	+
11. no anti- gen	—	—	—	—	—	—	—
12. 0.5 normal rabbit serum	—	—	—	—	—	—	—
13. 0.5 normal salt	—	—	—	—	—	—	—

TABLE 4

PRECIPITIN TEST: $\frac{3}{4}$ SAT. RABBIT IMMUNE SERUM. 0.1 CC ANTISERUM IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S. 2 CC IN ALL TUBES

Dilution of Antigen 1% Solution, C C	$\frac{3}{4}$ Saturation as Antigen	Egg white as Antigen
1. 0.5.....	+	+
2. 0.25.....	+	+
3. 0.125.....	+	+
4. 0.0625.....	+	+
5. 0.03125.....	+	+
6. 0.0156.....	+	+
7. 0.0078.....	Faint trace	+
8. 0.0039.....	Faint trace	+
9. 0.0019.....	Faint trace	No precipitation
10. 0.0009.....	Faint trace	No precipitation
11. no antigen.....	No precipitation	No precipitation
12. 0.5 + normal rabbit serum.....	No precipitation	No precipitation
13. 0.5 + normal salt.....	No precipitation	No precipitation

TABLE 5

PRECIPITIN TEST: $\frac{4}{4}$ SAT.-RABBIT-IMMUNE SERUM. 0.1 CC ANTISERUM IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S. 2 CC IN ALL TUBES

Dilution of Antigen 1% Solution, C C	$\frac{4}{4}$ Saturation as Antigen	Egg white as Antigen
1. 0.5.....	+++	++
2. 0.25.....	++	—
3. 0.125.....	+	—
4. 0.0625.....	—	—
5. 0.03125.....	—	—
6. 0.0156.....	—	—
7. 0.0078.....	—	—
8. 0.0039.....	—	—
9. 0.0019.....	—	—
10. 0.0009.....	—	—
11. no antigen.....	—	—
12. 0.5 + normal rabbit serum.....	—	—
13. 0.5 + normal salt.....	—	—

TABLE 6

COMPLEMENT FIXATION TESTS: $\frac{3}{4}$ SAT.-RABBIT-IMMUNE SERUM, 0.1 CC ANTISERUM (56 C., $\frac{1}{2}$ HOUR) IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S. 4 CC IN ALL TUBES

Antigen Dilution 1% Solution	$\frac{3}{4}$ Saturation as Antigen	Egg white as Antigen
1. 1:20.....	+++	+++
2. 1:40.....	+++	+++
3. 1:80.....	+++	+++
4. 1:160.....	+++	+++
5. 1:320.....	+++	+++
6. 1:640.....	+++	+++
7. 1:1,280.....	+++	+++
8. 1:2,560.....	+++	+++
9. 1:5,120.....	+++	+++
10. 1:10,240.....	Hemolysis complete	Hemolysis complete
11. Normal NaCl.....	Hemolysis complete	Hemolysis complete
12. 1:20 + 0.1 normal rabbit serum.....	Hemolysis complete	Hemolysis complete
13. 1:20 + normal NaCl.....	Hemolysis complete	Hemolysis complete

TABLE 7

COMPLEMENT FIXATION TESTS: 4/4 SAT.-RABBIT-IMMUNE-SERUM, 0.1 C C ANTISERUM (56 C.,
 $\frac{1}{2}$ HOUR) IN EACH OF FIRST 10 MIXTURES, NORMAL SALT SOLUTION Q. S.
 4 C C IN ALL TUBES

Antigen Dilution 1% Solution	4/4 Saturation as Antigen	Egg white as Antigen
1. 1:20.....	++++	++
2. 1:40.....	++	Hemolysis complete
3. 1:60.....	+	Hemolysis complete
4. 1:160.....	Slight inhibition	Hemolysis complete
5. 1:320.....	Slight inhibition	Hemolysis complete
6. 1:640.....	Slight inhibition	Hemolysis complete
7. 1:1,280.....	Slight inhibition	Hemolysis complete
8. 1:2,560.....	Slight inhibition	Hemolysis complete
9. 1:5,120.....	Hemolysis complete	Hemolysis complete
10. 1:10,240.....	Hemolysis complete	Hemolysis complete
11. Normal NaCl.....	Hemolysis complete	Hemolysis complete
12. 1:20 + 0.1 normal rabbit serum.....	Hemolysis complete	Hemolysis complete
13. 1:20 + normal NaCl.....	Hemolysis complete	Hemolysis complete

TABLE 8

ANAPHYLAXIS EXPERIMENTS WITH PROTEOSES FROM EGG WHITE*

Sensitizing Dose	Days Interval	Second Injection	Results	Subsequent Injec- tions
1. 0.010 gm. 1/4 sat.	21	0.025 gm. 1/4 sat.	No reaction	4 days later, 1 c c 50% egg white, no reaction
2. 0.010 gm. 1/4 sat.	21	0.025 gm. 1/4 sat.	No reaction	4 days later, 1 c c 50% egg white, no reaction
3. 0.010 gm. 1/4 sat.	21	0.025 gm. 1/4 sat.	No reaction	4 days later, 1 c c 50% egg white, no reaction
4. 0.010 gm. 1/3 sat.	21	0.025 gm. 1/3 sat.	No reaction	4 days later, 1 c c 50% egg white, sick in 15 min., recov- ered in 30 minutes
5. 0.010 gm. 1/3 sat.	21	0.025 gm. 1/3 sat.	No reaction	
6. 0.010 gm. 1/3 sat.	21	0.025 gm. 1/3 sat.	No reaction	
7. 0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
8. 0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
9. 0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
10. 0.010 gm. 1/2 sat.	21	0.025 gm. 1/2 sat.	No reaction	1 c c 50% egg white, no reaction
11. 0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
12. 0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
13. 0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
14. 0.010 gm. 2/3 sat.	21	0.025 gm. 2/3 sat.	No reaction	1 c c 50% egg white, no reaction
15. 0.010 gm. 3/4 sat.	23	0.025 gm. 3/4 sat.	Vigorous scratch- ing	1 c c 50% egg white, vigorous scratch- ing
16. 0.010 gm. 3/4 sat.	23	0.025 gm. 3/4 sat.	Vigorous scratch- ing, roughing of fur	1 c c 50% egg white, vigorous scratch- ing
17. 0.010 gm. 3/4 sat.	23	2 c c 10% egg white	Vigorous scratch- ing	1 c c 50% egg white, heaving respira- tion
18. 0.010 gm. 3/4 sat.	23	2 c c 10% egg white	Scratching	

TABLE 8—*Continued*
ANAPHYLAXIS EXPERIMENTS WITH PROTEOSES FROM EGG WHITE*

Sensitizing Dose	Days Interval	Second Injection	Results	Subsequent Injections
19. 0.010 gm. 4/4 sat.	23	0.025 gm. 4/4 sat.	Immediate convulsive breathing, recovery, 5 minutes	
20. 0.010 gm. 4/4 sat.	23	0.025 gm. 4/4 sat.	Roughing of fur, slight difficulty in breathing	
21. 0.010 gm. 4/4 sat.	23	2 cc 10% egg white	Scratching	1 cc 50% egg white, vigorous scratching
22. 0.010 gm. 4/4 sat.	23	2 cc 10% egg white	Scratching	1 cc 50% egg white, vigorous scratching
23. 2 cc 5% egg white	21	0.050 gm. 3/4 sat.	No reaction	1 cc 50% egg white, heaving resp.
24. 2 cc 5% egg white	21	0.050 gm. 3/4 sat.	No reaction	1 cc 50% egg white, vigorous scratching
25. 2 cc 5% egg white	21	0.050 gm. 4/4 sat.	No reaction	1 cc 50% egg white, vigorous scratching
26. 2 cc 5% egg white	21	0.050 gm. 4/4 sat.	No reaction	1 cc 50% egg white, no reaction
27. 2 cc 5% egg white	21	0.050 gm. 3/4 sat.	Vigorous scratching	1 cc 50% egg white, no reaction
28. 2 cc 5% egg white	21	0.050 gm. 3/4 sat.	Vigorous scratching	4 days later, 1 cc egg white, no reaction
29. 2 cc 5% egg white	21	2 cc 25% egg white	Exitus, typical shock	
30. 2 cc 5% egg white	25	2 cc 25% egg white	Exitus, typical shock	

* The sensitizing injections were given intraperitoneally, the intoxicating doses, intracardially. The various substances tested proved non-toxic for normal animals in the doses employed.

Anaphylaxis experiments with guinea-pigs served to confirm the lack of any antigenic properties possessed by the $\frac{1}{4}$, $\frac{1}{3}$, $\frac{1}{2}$ and $\frac{2}{3}$ ammonium sulphate saturated fractions, and showed that the $\frac{3}{4}$ and $\frac{4}{4}$ saturated products possessed slight sensitizing and intoxicating properties, the latter being apparently the more active.

In all forms of antibody production the antigenic proteoses were not specific, in that egg white could be used as antigen in place of either fraction when carrying out the tests. The reverse was not so marked.

(c) *Toxin-Antitoxin Reaction*.—According to Mitchell and Reichert⁷⁴ (1883) cobra venom contains 2% globulin which is the hemolysin and 98% of substances resembling "peptone," by which they probably meant substances now classed as proteoses. Antitoxin against cobra venom is quite efficient, and the possibility remains that we are dealing here with an anti-albumose. A further possibility is suggested, namely, are whole proteins necessary for anaphylaxis, precipitins and complement binding antibodies, while antitoxins are the result

⁷⁴ Cited by Wells, *Chemical Pathology*, 1918, p. 148.

of antigens consisting of derived proteins-albumoses? Weichardt⁷⁵ in connection with his studies of fatigue toxins (kenotoxins) obtained considerable evidence that the substances against which he was able to produce antitoxins represented the first stages in the disintegration of the protein molecule, and therefore closely related to proteoses.

One form of the epiphanin reaction reported by Weichardt⁷⁶ seemed to furnish a method of demonstrating the toxin-antitoxin reaction in vitro. He found that catalyzers (hemoglobin, platinum black) are markedly affected by bacterial toxins. If a water soluble toxin such as tetanus toxin be added to a much diluted blood, allowed to stand at 37 C. for 30 minutes, and then tincture of guaiac and hydrogen peroxid be added, the well known guaiac reaction does not occur. If the toxin is previously treated with its specific antitoxin there is no interference with the guaiac reaction and the solution turns blue. Nonspecific reactions may be avoided by quantitative means (dilution of antigen and serum). In addition to toxins, products derived from proteins, including kenotoxin, also inhibited the guaiac reaction.

I have tested Weichardt's reaction, using the detailed protocol contained in his article. Diphtheria toxin and antitoxin, tetanus toxin and antitoxin, and all our proteose fractions and their corresponding serums were used. In the case of the bacterial toxins relatively large amounts of toxin were required for inhibition (500 units of diphtheria toxin and 1,000 of tetanus toxin). Much larger quantities of antitoxin were required to neutralize their action. The proteose preparations even as much as 0.1 gm., did not give the characteristic reaction. In my experience, the reaction was not of sufficient delicacy to be used in any sense as a quantitative test for the determination of toxin-antitoxin reactions.

SUMMARY

The results of these experiments indicate that Gibson's alcohol-soluble proteose obtained from Witte "peptone" is unable to stimulate the production of precipitins or complement binding antibodies when injected into rabbits. Two other fractions obtained from Witte "peptone" yielded similar results.

Anaphylaxis experiments with guinea-pigs showed that the Witte "peptone" preparations possessed only very slight power of sensitizing to themselves. They are not able to sensitize to beef serum, nor do they produce symptoms of anaphylactic intoxication in animals sensitized to beef serum. Beef serum did not sensitize to products of Witte "peptone," nor did it produce "shock" in animals sensitized to such products.

⁷⁵ Ueber Ermüdungsstoffe, 1910.

⁷⁶ Münch. med. Wehnschr., 1911, 58, p. 1662.

Proteose preparations were obtained by hydrolysis of coagulated egg white and fractional precipitation with ammonium sulphate in the manner described. There seems to be ground for disagreement with Haslam when he claims that constant nitrogen values as shown by Kjeldahl determinations are an index of purity of proteose preparations. A mixture of proteoses which is constant in its proportions will show constant quantities of nitrogen. Kjeldahl nitrogen determinations of my preparations showed a maximum difference of less than 2% between all of them. When injected into dogs they showed the characteristic physiologic action of proteoses.

Experiments with rabbits indicated that the $\frac{1}{4}$, $\frac{1}{3}$, $\frac{1}{2}$ and $\frac{2}{3}$ saturation products possessed no power of stimulating the production of precipitins or complement binding antibodies. They also were unable to sensitize or intoxicate guinea-pigs either to themselves or to egg white, the mother protein.

The $\frac{3}{4}$ and $\frac{4}{4}$ saturation products were not as efficient antibody producers as egg white, but showed definite antigenic power for both precipitins and complement binding substances. In guinea-pigs the $\frac{3}{4}$ and $\frac{4}{4}$ saturated products possessed slight sensitizing and intoxicating properties, the latter being apparently the more active.

In all forms of antibody reaction the antigenic proteoses were not specific, in that egg white could be used as antigen in place of either fraction. The converse was not so marked.

Experiments with Weichardt's epiphanin reaction (interference with the guaiac blue reaction of blood) indicated that it is not sufficiently delicate to be used as a quantitative determination of the toxin-antitoxin reaction in vitro.

If the immune reactions are reliable indicators of chemical relationships, these results would seem to indicate that the $\frac{3}{4}$ and $\frac{4}{4}$ saturated fractions are chemically but little different from the mother substance, or at least contain similar antigenic groups. This is of special interest in view of the fact that the first fractions to be precipitated are usually considered the larger molecules and most closely related to the original proteins.

THE EFFECT OF POTASSIUM IODID ON EXPERIMENTAL SPOROTRICHOSIS

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The relatively large number of human cases of sporotrichosis now observed has furnished abundant opportunity for testing the effect of potassium iodid on the course of this infection. Almost without exception such cases respond promptly to its administration in doses of 30-40 grains per day and usually a complete cure follows in the course of a few weeks. Its administration is continued some little time after complete healing is apparent since otherwise a recurrence is apt to follow. It has also been shown by Carongeau¹ that horses and mules, which are naturally susceptible to this disease, likewise respond promptly to this drug. The results, on the whole, show that the iodids in sporotrichosis furnish one of the best examples of a specific therapeutic agent known.

One is impressed with the difference between the prompt reaction to potassium iodid of sporotrichotic patients and the effect of potassium iodid in other similar infections like blastomycosis, actinomycosis and tuberculosis, in which cases the reaction is irregular and uncertain and may be beneficial, indifferent, or possibly harmful.

I wish to record in this paper certain experiments to test the action of the iodids on the course of experimental infection in animals produced with typical strains of *Sporotrichum schenckii*.

First, I will call attention to the relative inertness of potassium iodid and of iodine so far as their direct germicidal power on sporotricha is concerned as illustrated in table 1.

It will be observed that potassium iodid has very little direct effect on the life of *Sporotrichum schenckii*. In a 10% solution the organisms live for at least 48 hours and in the 1% solution and in those of lesser concentration the organisms were alive at the end of 74 days at which time this experiment was discontinued. In distilled water they were also alive at the end of this time. A strain of

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¹ Quoted from de Beurmann et Gougerot, *Les Sporotrichoses*, Paris, 1912.

Sporotrichum schenckii was used which had been isolated from a human lesion a few months previously and at the time was pathogenic for rats. The culture was a vigorous growing one about 4 weeks old. At the varying intervals indicated in the table, 0.1 cc of the sporotrichum suspension was removed from each of the tubes and inoculated on glucose agar and the readings made after 3 weeks.

TABLE 1
GERMICIDAL ACTION OF AQUEOUS SOLUTION OF POTASSIUM IODID ON SPOROTRICHUM SCHENCKII (CULTURE ONE MONTH OLD)

Time	0.1% K I	0.5% K I	1% K I	2% K I	5% K I	10% K I	Water
1 minute	+++	+++	+++	+++	+++	+++	+++
15 minutes	+++	+++	+++	+++	+++	+++	+++
1 hour	+++	+++	+++	+++	+++	++	+++
24 hours	++	++	++	+++	++	++	++
48 hours	++	++	++	++	++	+	+++
96 hours	++	++	++	++	++	0	++
5 days	++	++	++	+	+	0	++
8 days	++	++	++	++	++	0	++
11 days	+++	+++	+++	+	++	0	+++
17 days	++	+++	+++	0	0	0	+++
23 days	++	++	++	++	0	0	++
32 days	++	++	++	++	0	0	++
47 days	++	++	++	0	0	0	++
56 days	++	++	++	0	0	0	++
74 days	++	++	++	0	0	0	++

These results are sufficient to indicate that iodine either in the form of potassium iodide or in a free form in the culture medium has very little germicidal effect on this fungus even in relatively high concentrations. The concentrations which the iodine attains in the body when administered therapeutically probably does not approach the concentrations in which the growth is inhibited in experiment, and could therefore, through its direct action, have no appreciable effect.

TABLE 2
EFFECT OF POTASSIUM IODIDE AND TINCTURE OF IODINE IN 1% GLUCOSE AGAR ON THE GROWTH OF SPOROTRICHUM SCHENCKII AND A BLASTOMYCES

Percentage of Potassium Iodide and Tincture of Iodine	Sporotrichum schenckii		Blastomyces		Schenk-Hektoen Strain (original)
	Tincture of Iodine	Potassium Iodide	Tincture of Iodine	Potassium Iodide	
10	0	++	0	+ (slight)	0
5	0	+++	0	++	0
4	0	+++	0	+++	0
3	0	+++	0	+++	0
2	0	+++	0	+++	0
1	0	+++	+	+++	+
0.5	++	+++	++	+++	+
0.2	++	+++	+++	+++	++
0.1	+++	+++	+++	+++	+++

Another experiment was made by adding potassium iodid and the tincture of iodine in varying concentration to a series of tubes containing 1% glucose agar. Table 2 gives the results. It will be seen that even in as high a concentration as 10% of potassium iodid the organisms grew very well and in all concentrations below that amount there developed a profuse growth. In the medium containing tincture of iodine one strain of *sporothrix* grew in 1% concentration and below, another grew at 5% and below. In 2% strengths and above there was no growth.

To throw further light on the possible mechanism of the action of potassium iodid in this infection a series of white rats approximately 100 gm. in weight was given the drug subcutaneously before, simultaneously with and after inoculation with a virulent strain of *Sporothrichum schenckii*. The strain had recently been isolated from a typical human case of sporotrichosis and was a good pigment producer and virulent. The sporotrichum was grown for 3 weeks on glucose agar, the growth was scraped from the surface and macerated in salt solution thereby obtaining a turbid suspension of spores and mycelium. In the following experiments 1 cc of this suspension was used uniformly in all the animals inoculated in the different series so that the experiments would be comparable.

1.—On March 12, two white rats were inoculated intraperitoneally as controls. April 10, small nodes on abdomen at points of inoculation. Nodules in abdomen large; animals not lively, and visibly affected. April 30, both killed; a large firm nodule in abdominal wall at the point of inoculation; peritoneum studded especially on the omentum, mesentery and bowel with numerous gray, firm nodules. Liver and lungs not involved. In smears of the crushed nodules were seen the oblong tissue forms and in cultures of this material numerous colonies of sporotricha appeared after a few days. Both rats presented almost identical findings. Other experiments previously made had likewise shown this particular strain of sporotrichum to be uniformly virulent for rats.

2.—Two white rats were given subcutaneously 0.05 gm. of a solution of potassium iodid each day for 8 successive days. On the last day (March 12) an intraperitoneal inoculation of 1 cc of the sporotrichum was given also. The potassium iodid was now discontinued. On April 10 the animals were in fair condition but somewhat thin. On April 28 both animals had large swollen, nodular, fixed testicles and one had nodes on the abdominal wall. One died May 18, the other on June 5. Both revealed similar findings, namely, extensive nodular lesions of peritoneum and viscera and extensive involvement of the bones of front and hind extremities. One weighed 60 gm., the other 72 gm. Cultures of heart blood were sterile. Cultures of the nodes and bones were profusely positive. The infection in these animals following the use of potassium iodid made as rapid, possibly a little more rapid, progress than in those of the control series.

3.—On March 12, two rats were each injected intraperitoneally with the sporotrichum and at the same time and on each of 6 successive days 0.05 gm. of potassium iodid was given, also intraperitoneally. Up to April 28 no symptoms, but at that time they showed some evidence of weakness and emaciation. One died on July 9, and showed typical sporotrichotic nodules on various parts of omentum, peritoneum and intestine. The nodes were softened; and smears and cultures from the interior showed many sporotricha. On July 12, the other rat being weak and sickly was chloroformed and revealed findings almost identical with the first rat. Evidently, then, the administration, simultaneously and on 6 successive days, was not sufficient to protect the rats, the infection going on very much the same as in the controls.

4.—On March 12, two rats were given intraperitoneally, injections of sporotricha and one month later (April 11) were given 0.05 gm. of potassium iodid continuously each day (excepting Sundays) subcutaneously. Shortly after the injection the rats became sickly and weak, and on April 10 their testicles were large, nodular and red. Both were evidently quite ill. Following the iodid, however, they soon improved gradually, and on April 28 seemed quite well excepting that one was somewhat emaciated, having lost about 15 gm. The testicles were much smaller but were nodular and firm. On May 7, one of the rats was found dead; cause of death undetermined. A few small, hard, white nodes in the mesentery and about the testicles were found. The involvement was not extensive; other viscera not involved. A few of these firm nodes from the mesentery were incised and crushed and cultures made yielded a decided growth. The heart blood was sterile. On June 4 the second rat having had potassium iodid continuously was quite well and was killed. Necropsy revealed similar nodules, hard, firm and small, especially about the testicles. A few small nodes were found also about the hind limb bones. The gross appearance of these nodes was that of a healing lesion. On cultures, however, the crushed nodes of the bone and peritoneum yielded a good growth of sporotricha. Evidently the lesions in these animals were healing though the organisms still remained alive within.

5.—Two white rats, on March 12, were injected with 1 cc intraperitoneally and at the same time with 1 cc subcutaneously just above the tail. About three weeks later, on April 4, nodules appeared on the belly at the site of inoculation, and over the tail was a large open ulcer 1.5 cm. across and with raised margins. Cultures from the deeper parts yielded a profuse growth of sporotricha. At this date 0.05 gm. doses of potassium iodid were commenced subcutaneously and continued daily. On April 9, four days later, the lesion over the tail was softer and smaller and on one rat a scab was forming. On April 14, the lesions in both animals were rapidly healing but cultures from them were still profusely positive. At this time 1 cc of culture of sporotricha was injected into each rat under the skin of the neck near the right shoulder; also about 2 drops under the skin of the tail about 3 cm. from its base. The potassium iodid was continued. The old lesion above the tail became smaller gradually but at the site of the new injections the lesions appeared which were red and swollen and edematous; they continued, both in the neck and on the tail of each rat, to progress for about 3 weeks then gradually receded. During this period, then at a time when the animal was receiving potassium iodid, the old lesions were healing, whereas the fresh lesions were progressing. The animals continued to improve and later all the lesions showed healing. On June 21, nine weeks after the last injections, the cutaneous lesions were practically well and from then until

July 10 the potassium iodid was given every other day, at which time the animals were killed, having been under observation for 4 months. They appeared quite normal and were not emaciated. On necropsy, both animals presented hard, small, whitish gray nodes in the peritoneum and mesentery about 0.1 mm. or less in diameter. On section these small lesions revealed a slight softening and on smear and culture sporotricha were obtained in considerable numbers. Also in one of the rats at the site of the neck lesion was a small node just visible which earlier had been as large as a cherry. This lesion had contracted and healed, but cultures from its center still revealed live sporotricha in considerable numbers. The lesions in the skin on and above the tail had practically disappeared.

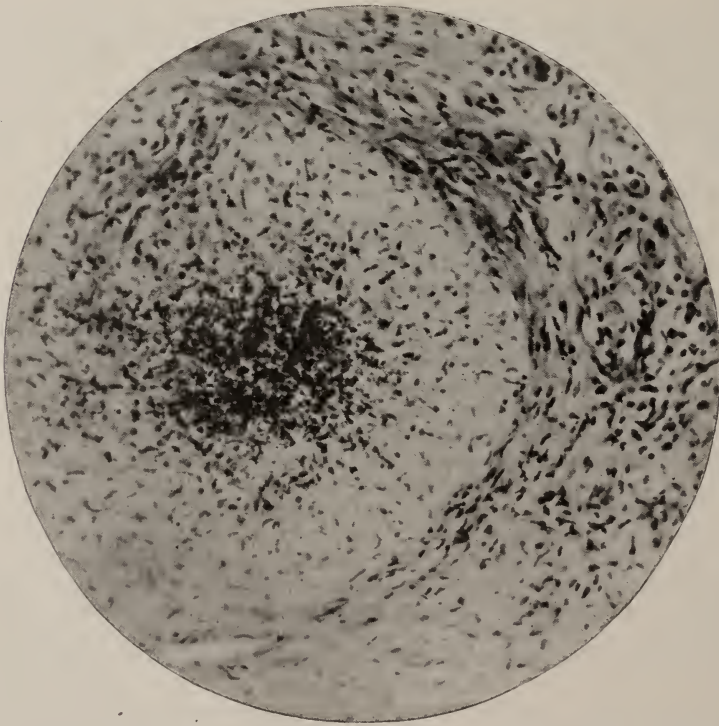


Fig. 1.—Encapsulated sporotrichotic nodule from the omentum of a rat following administration of potassium iodid.

Sections were made of these hard lesions, a photograph of which is shown in Fig. 1. The dense fibrous capsule is seen and in the center is still some necrotic tissue in which occur the organisms. In Fig. 2 are to be seen small recent sporothrix lesions in the liver of a rat for comparison.

It would appear that the lesions under the influence of the potassium iodid become smaller and harder and a marked increase in connective tissue occurs which tends to wall off the organisms. However, the potassium iodid does not appear to have any appreciable

direct effect in killing sporotricha for they were still found in centers of the hardened nodules several months later. This is in accord with the experimental data presented earlier in the paper which showed little or no direct action on the organisms.

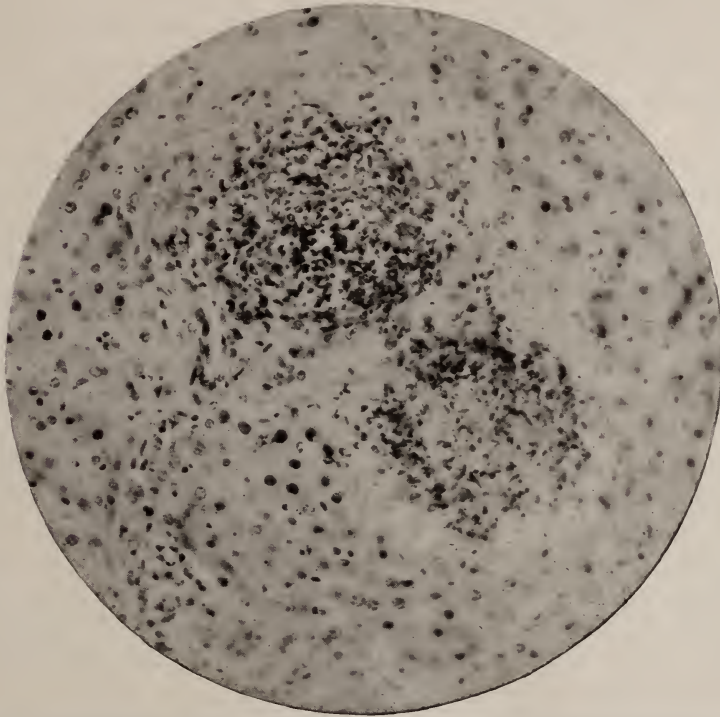


Fig. 2.—Sporotrichotic nodule in the liver of a white rat suffering from a progressive sporotrichosis without treatment.

DISCUSSION

The experiments indicate that potassium iodid given to animals for 8 days previous to inoculation with sporotricha will have no effect in inhibiting or preventing the infection. Also, when given simultaneously and for a week following the inoculation, the infection appears to continue without interruption. When the potassium iodid is continued indefinitely the lesions heal becoming hard and contracted, but the sporotricha remain alive in their centers for a long time and apparently are unable to disseminate.

Lesions following inoculation under the skin usually progress, but if potassium iodid is administered they gradually recede; such lesions

can be inspected from day to day and the healing process observed. By reinoculating such an animal, even when receiving potassium iodid, the new infection will progress for a period of 2 or 3 weeks and then recede so that it is possible to have in one and the same animal, one lesion which is healing under the influence of potassium iodid and another which is progressing. Evidently the potassium iodid does not act in a direct way on the sporotricha, but indirectly through the agency of tissue proliferation and processes incidental thereto.

From the experiments it seems clear that potassium iodid acts in such a way as to stimulate the healing process without inhibiting the development of the infecting organism. In other words, its action is curative and not preventive. De Beurmann and Gougerot have pointed out practically the same fact in connection with their studies of *Sporotrichum beurmanni*, their experiments also indicating that potassium iodid acts in a curative rather than in a preventive way. They contend, however, that *Sporotrichum schenckii* is different from *Sporotrichum beurmanni*. Without here entering into a discussion of this point, it may be stated that many facts indicate their identity including the reaction of the two infections to potassium iodid not only in animals but in human cases.

In my experiments potassium iodid was used, but other compounds of iodine or free iodine would probably behave in the same way. Sollmann² has shown that no matter in what form iodine is given it circulates in the blood and is excreted in the form of the iodid. He also states, what is important in connection with the action of potassium iodid on sporotrichum infections, that it is impossible under any conditions existing in the body (excepting solely the gastric juice) for free iodine to be liberated from iodid. This is in entire accord with the results of my experiments, all of which tend to show that iodine does not act directly on the organisms.

As to other possible mechanisms by which iodine acts in the body that developed by Lortat-Jacob³ and advocated by many French workers is interesting in this connection. Lortat-Jacob contends that the leukocytes are the active agents in the absorption of iodids or iodine after peritoneal or subcutaneous injection. The iodine can actually be seen coloring the cells and may be detected by the starch method. After peritoneal injection an endothelial reaction with

² Cleveland Med. Jour., 1916, 15, p. 792.

³ Thèse, 1903, Paris.

accumulation of endothelial leukocytes occurs. He maintains that this reaction, especially of the mononuclear and lymphoid tissue, by their defensive properties permits an explanation of the good effects of iodine in chronic infections. He says that under the influence of iodine there is congestion and hyperactivity of the lymphoid tissue and repeated small doses may lead to sclerosis of tissues. The French, it is interesting to note, have used and continue to use iodine in tuberculosis especially of the glandular type with what they consider encouraging results.

Wells and Hodenberg⁴ have shown that tuberculous as well as other necrotic tissues take up more iodine than normal tissue because the dead cells are more permeable to iodids. But they obtain no evidence that iodine tends to become specifically fixed in inflammatory exudates. It is distributed by a process of simple diffusion. Heinz⁵ noted an active exudative inflammation following the injection of iodine in the body cavities and Hirsch⁶ observed an increased fibrous tissue reaction about masses of fat-free tubercle bacilli treated with an iodine solution. Jobling and Peterson⁷ have called attention to the possible rôle of iodine in tuberculosis and other infections in saturating the unsaturated fatty acid soaps which act as antiferments, thereby permitting the tryptic enzymes to digest the cells and tissues and to remove caseous matter. This increased ferment activity may play a rôle in the cure of sporotrichosis by iodids. Natural or experimental sporotrichosis might furnish excellent material for testing this hypothesis further.

SUMMARY

Experimental sporotrichosis in rats responds promptly to potassium iodid.

The lesions in the peritoneal cavity become firm, hard and small and are surrounded by a dense fibrous capsule; within the nodules living sporotricha are found for a long time (at least 4 months).

Potassium iodid will not prevent experimental sporotrichosis but will cure it.

It is suggested that sporotrichosis, so readily produced experimentally, furnishes a good opportunity for the study of the behavior and reactions of iodine and iodids in chronic infections.

⁴ Jour. Infect. Dis., 1912, 11, p. 439.

⁵ Virchow's Archiv, 1899, 155, p. 44.

⁶ Jour. Infect. Dis., 1914, 15, p. 487.

⁷ Jour. Exper. Med., 1914, 19, p. 383.

THE MICROSCOPIC APPEARANCES IN ULCEROMEMBRANOUS TONSILLITIS (VINCENT'S ANGINA)

With one plate

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The etiologic relation of fusiform bacilli and the associated spiral organisms now commonly called spirochetes, to the diseases in which they are found is difficult of demonstration on account of the presence of streptococci and other bacteria in cultures; and then the bacilli and spirals so far isolated usually lack virulence for animals. The strongest evidence of an etiologic relation has been furnished by the examination of sections of the involved tissues. Many observers have demonstrated these organisms in noma,¹ at the line of advancing necrosis, where they are found in great numbers, apparently pure, and also in small numbers in the unaltered tissue. The spiral forms have generally been observed in the living tissue in advance of the bacilli (Perthes,² Buday³ and Ellermann⁴).

Vincent,⁵ Krebs,⁶ and Gross⁷ have studied sections of the ulcer-membranous lesions of the tonsils, and Ellermann⁸ of the uvula. They observed a variety of bacteria, especially cocci in the external necrotic layer; in the middle zone between necrotic and normal tissue a large number of fusiform bacilli, often in palisade arrangement, generally not associated with other bacteria; and a few bacilli alone invading the living tissue, in strands. The inflammation was described as fibrinous, and a variable amount of leukocytic infiltration was observed. Vincent and Ellermann found no spiral forms in the sections; Gross found only a few.

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¹ For a review of the literature see Weaver and Tunnicliff: *Jour. Infect. Dis.*, 1907, 4, p. 8.

² *Archiv. f. klin. Chir.*, 1899, 59, p. 111.

³ *Beitrage z. path. Anat. u. z. allgem. Path.*, Ziegler, 1905, 38, p. 255.

⁴ *Centralbl. f. Bakteriol.*, I, O., 1905, 38, p. 383.

⁵ *Annal. Inst. Pasteur*, 1899, 13, p. 608.

⁶ *Deutsch. Med. Wchnschr.*, 1902, 28, p. 310.

⁷ *Deutsch. Arch. f. klin. Med.*, 1904, 79, p. 369.

⁸ *Centralbl. f. Bakteriol.*, I, O., 1905, 38, p. 383.

The ulceromembranous tonsil, described in this report, was removed during life, and the removal proved an effective method of limiting the infection. The case was clinically typical of Vincent's angina. Smears showed large numbers of fusiform bacilli and long spiral organisms with wide undulations.

Sections were stained by Levaditi's method to demonstrate the spiral forms, which are not readily stained by other methods. These sections show the tonsil covered with an exudate (not seen in the figures) containing cocci, a variety of bacilli and a few spiral organisms; under the exudate the external epithelium is necrotic and contains only an occasional coccus, fusiform bacillus and spiral form (Fig. 1, A). The layer between this necrotic tissue and living tissue contains a few leukocytes, a moderate amount of fibrin, and enormous numbers of fusiform and spiral organisms (Fig. 1, B). Their presence in the deeper tissues is probably due to their being anaerobic organisms. The microphotographs give no evidence of the large number of spiral forms present on account of their not lying in one plane, and therefore not easily photographed. The bacilli are often packed together side by side in palisade arrangement. The necrotic lymph follicles are replaced by a mass of fusiform bacilli and spirals, the latter being almost pure in the center of the follicle, the bacilli radiating from them. Living tissue is seen beneath this mass of organisms (Fig. 1, C). The fusiform and spiral organisms invade the living tissue, the latter generally just ahead or considerably in advance of the bacilli (Figs. 2, 3 and 4), being here more numerous than the bacilli.

Sections stained with methylene blue and the Gram-Weigert method show cocci only in the exudate and external layer of the tonsil and not associated with the fusiform bacilli and spirals near and in the living tissue. The coccus-like bodies seen in Figure 3 are sections of the fusospirillar organisms. The bacillary elements, especially the granules, retain the gram stain somewhat, the spirillar elements are completely decolorized.

SUMMARY

Sections of an ulceromembranous tonsil show fusiform bacilli and spiral organisms in enormous numbers in the zone separating the external necrotic epithelium and living tissue. A considerable number are invading the normal tissue, the spirillar forms being the more abundant and in advance of the bacilli. These organisms are apparently not associated with cocci or other bacteria at the line of advancing necrosis.

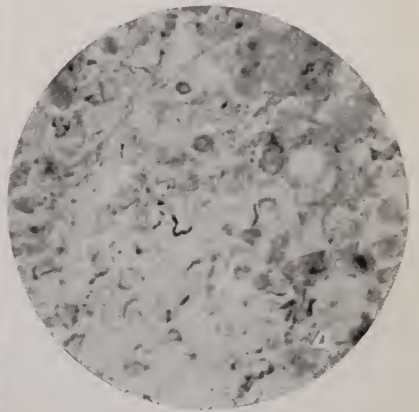
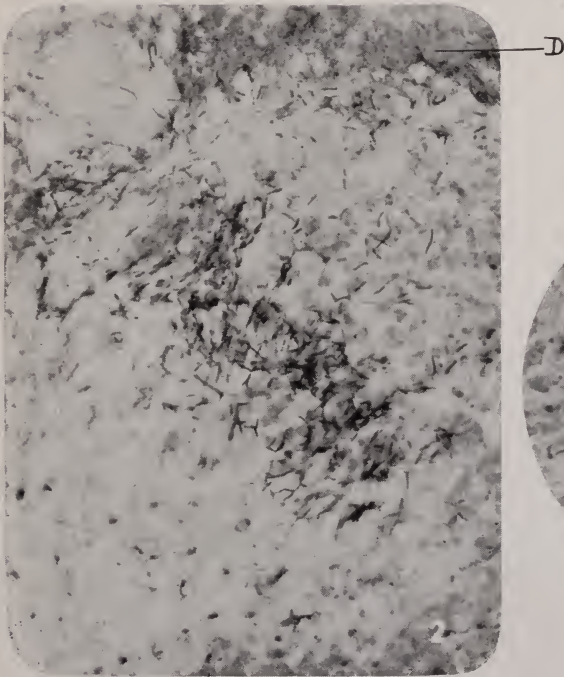
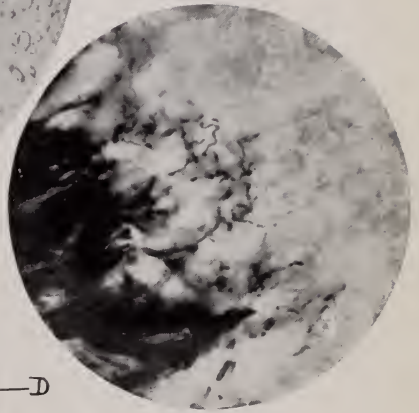
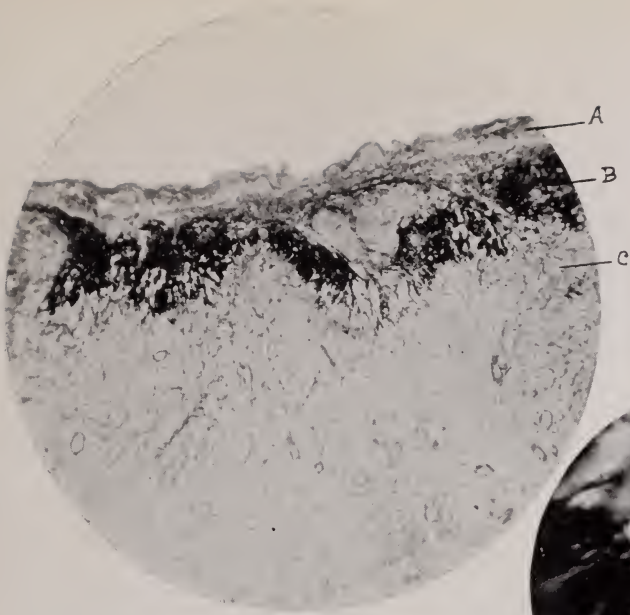
EXPLANATION OF PLATE

Fig. 1.—Section through tonsil showing external necrotic layer (*A*) containing practically no bacteria; middle layer (*B*) made up of masses of fusiform bacilli and spirochetal forms; living tissue (*C*). Levaditi preparation $\times 90$.

Fig. 2.—Section through edge of middle layer showing the organisms invading living tissue. The spiral forms are in advance of the bacilli. *D* is a mass of spiral organisms. Levaditi preparation $\times 575$.

Fig. 3.—Section showing spiral organisms invading living tissue just in advance of the bacilli. Levaditi preparation $\times 1200$.

Fig. 4.—Section showing spiral forms in the living tissue, represented by *C* on figure 1. Levaditi preparation $\times 1200$.



THE DIFFERENTIATION AND DISTRIBUTION OF THE PARATYPHOID-ENTERITIDIS GROUP. VI

AVIAN PARATYPHOID BACILLI: A COMPARATIVE STUDY OF *B. PULLORUM* AND *B. SANGUINARIUM*

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Two organisms of the paratyphoid-enteritidis group designated, respectively, as *B. pullorum* and *B. sanguinarium*, are considered responsible for certain diseases of the domestic fowl. They are generally regarded as quite widely distributed thruout the United States. We have, nevertheless, little definite knowledge at present as to the prevalence of the diseases caused by these organisms. Smith and Ten Broeck¹ have suggested that *B. sanguinarium* may be the cause of the occasional reports of gastro-intestinal disturbances following the eating of meat of fowls. The feeding experiments of Rettger, Hull and Sturges² suggest that *B. pullorum* may be the cause of food poisoning. The disease in fowls produced by *B. sanguinarium* resembles clinically that caused by *B. avisepticus*, and Hadley³ states that many of the laboratory cultures of *B. avisepticus* are really *B. gallinarum* or some member of the paratyphoid-enteritidis group. Several investigators have observed the close relationship of these organisms to each other, their antigenic and cultural relations to *B. typhosus*, and the general relationship of these organisms to the paratyphoid-enteritidis group. In view of these facts it appears that further study of these organisms, is quite desirable.

B. sanguinarium was first isolated in the United States in 1894 by Smith,¹ from an epidemic among fowls in Rhode Island. Moore⁴ isolated the same organism in 1895 from diseased fowls in Washington, D. C., Virginia, and Maryland and gave the first detailed description of the organism and the disease produced by it. He at first called the disease "infectious leukemia" because of the reduction in number of the red blood cells and the increase in the number of white blood cells. Curtis⁵ studied an outbreak of the disease in Rhode Island in 1901 and called the disease "fowl typhoid," which is the name accepted today. *B. sanguinarium* has been isolated more recently

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¹ Jour. Med. Research, 1915, 31, p. 503.

² Jour. Exper. Med., 1916, 23, p. 475.

³ Jour. Bacteriol., 1918, 3, p. 286.

⁴ Twelfth and Thirteenth Annual Reports, Bur. An. Ind., U. S. Dept. of Agric., p. 185.

⁵ R. I. Exper. Sta., Bull. 87, 1902.

from several epidemics in the New England states. Pfeiler and Rehse⁶ in 1913 described what was apparently the same organism as the cause of an epidemic among fowls in Germany. They named it *B. typhi gallinarum alcalifaciens*, apparently not knowing that it had been previously described by Moore and others. Pfeiler and Roepke⁷ have isolated this organism from a second epidemic in Germany and have given quite a thoro description of it, which shows that it is undoubtedly the same organism described by Moore. Lignières and Zabala⁸ isolated a paratyphoid-like organism from fowls during an epidemic among fowls in South America and from their description, the bacterium appears to be very similar if not identical to *B. sanguinarium*. It also appears that Klein,⁹ as has been suggested by Hadley in a personal communication, described the same organism in 1889 under the name *B. gallinarum* as the cause of an epidemic in England. Altho his description is not as complete as has been given in recent years by Moore and others, yet his description and my own work with this organism indicate that it is identical with the one described by Moore. Since the organism studied by Klein apparently has the priority in nomenclature, it appears that the name of the bacillus of fowl typhoid should be *B. gallinarum*. Final decision on this point, however, should await a classificatory revision of the whole group. In order to avoid confusion at this time I shall use the name *B. sanguinarium*.

B. pullorum as the cause of the disease known as "bacillary white diarrhea" was first isolated in 1899 by Rettger¹⁰ from young chicks which had died of the disease. There has been some doubt, at times, as to whether this organism was really the cause of white diarrhea of chicks. Morse¹¹ in 1909 observed that this disease was due to a coccidium. A recent Farmers' Bulletin (957) issued by the Bureau of Animal Industry, Washington, D. C., states that a clinical disease called "white diarrhea" of chicks may be caused by *B. pullorum*, *B. avisepticus*, a coccidium, or *Aspergillus fumigatus*. During the past few years *B. pullorum* has been the cause of epidemics among chicks in many parts of the United States. It has been observed, however, much more frequently in the New England states. Young chicks under 72 hours of age are considered to be especially susceptible, altho Jones¹² and Hadley¹³ have described epidemics among adult fowls, due to this organism.

The pathologic changes produced by *B. pullorum* have been thoroly studied by Gage and Martin.¹⁴ They state that the disease in small chicks is a septicemia and that the minute necrotic foci in the liver, spleen, and pancreas are the most characteristic lesions. If the chick recovers from an infection it may become a permanent carrier. Horton¹⁵ reports the apparent recovery of a bantam hen which evidently had an ovarian infection, since two of her eggs contained the organism and the agglutination test with her serum was positive; however, a year later the organisms could not be isolated from the eggs and the agglutination reaction was negative. The ovary seems to be the most

⁶ Mitth. d. Kaiser-Wilhelms Inst. f. Landwirtschaft in Bromberg, 1913, No. 4, p. 306.

⁷ Centralbl. f. Bakteriöl., 1917, 79, p. 125. (Abstracted in Jour. Comp. Path. and Therap., 1917, 30, p. 263.)

⁸ Hoare, A System of Veterinary Med., 1913, 1, p. 466.

⁹ Centralbl. f. Bakteriöl., 1889, 5, p. 689.

¹⁰ N. Y. Med. Jour., 1900, 71, p. 803.

¹¹ U. S. Dept. of Agric., B. A. I. Circular 128 (1909).

¹² Jour. Med. Research, 1913, 27, p. 471.

¹³ R. I. Exper. Sta., Bull. 172.

¹⁴ Jour. Med. Research, 1916, 34, p. 149.

¹⁵ Jour. Bacteriöl., 1916, 1, p. 625.

frequent site of the infection in adult hens and often has a pathologic appearance. However, apparently normal ova may carry the organism to the full formed egg.

In the present study of *B. pullorum* and *B. sanguinarium* comparisons have been made with typical strains of members of the paratyphoid-enteritidis group, and with *B. typhosus* and *B. avisepticus*. Typical representatives of the paratyphoid-enteritidis group were obtained from Jordan¹⁶ who has made an extensive study of this group. For the history of these strains the reader may refer to his publications. Typical strains of *B. typhosus* were taken from stock cultures. It was somewhat more difficult to secure typical strains of *B. avisepticus*, hence it was necessary to devote some time to the study of this organism. Altho an organism was described under this name as early as 1879 by Toussaint and in 1880 by Pasteur, yet there are very few data concerning its fermentative action on the carbohydrates and, furthermore, there is considerable disagreement as to many of its characteristics, such as fermentative action, indol production, and pathogenesis. It is generally agreed, however, that this organism is a short gram-negative, nonmotile rod which does not liquefy gelatin. It is usually described as having a marked tendency to stain deeper at the poles, especially when stained from the blood of an infected fowl. The tendency to polar staining, however, is markedly reduced when grown on artificial mediums or when inoculated into rabbits; hence it is often quite difficult to distinguish it from *B. sanguinarium*, which frequently stains lightly in the central part. *B. avisepticus* is usually highly virulent for rabbits, but may soon lose this virulence when grown on artificial mediums. This may explain in part the contradictory results observed by various investigators concerning its pathogenesis. In view of these facts it seemed advisable to include the reaction of strains of *B. avisepticus* in the tables and in certain discussions.

The organisms which have been studied in the present work were obtained from as widely different sources as possible and were tested for purity by plating on Endo medium. Three colonies were picked from the Endo plates and compared with the original, in milk containing brom-cresol-purple as an indicator and in dextrose, lactose and maltose broth fermentation tubes containing an indicator to show any change in the acidity as well as gas production. The source of the cultures is given in table 1.

¹⁶ Jour. Infect. Dis., 1917, 20, p. 457.

TABLE 1
SOURCE OF CULTURE

Strain	Original Label	Received from	Isolation		
			Date	Location	By whom
B. sanguinarium 1	B. of Fowl typhoid I	Dr. Theobald Smith...	1895	Va. or Md.	Dr. V. A. Moore
2	B. of Fowl typhoid II	Dr. Theobald Smith...	1894	R. I.	Dr. Theobald Smith
3	B. sanguinarium 781	Am. Mus. Nat. Hist. ..	Mar. '17	N. Y.	Dr. Chas. Krumweide
11	B. sanguinarium "Taylor"	Dr. W. A. Hagan.....	Apr. '14	Calif.	Dr. W. J. Taylor
12	B. sanguinarium "Smith IV"	Dr. W. A. Hagan.....	Dr. Theobald Smith
13	B. gallinarum No. 51 or Rettgers cult. "S"	Dr. P. B. Hadley.....	Conn.	Dr. L. F. Rettger
14	B. gallinarum No. 108	Dr. P. B. Hadley.....	Oct. '14	R. I.	Dr. P. B. Hadley
15	B. gallinarum No. 110	Dr. P. B. Hadley.....	Oct. '14	R. I.	Dr. P. B. Hadley
50	B. gallinarum No. 42	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
51	B. gallinarum No. 45	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
52	B. gallinarum No. 66	Dr. P. B. Hadley.....	Conn.	Dr. P. B. Hadley
53	B. gallinarum No. 69	Dr. P. B. Hadley.....	N. Y.
54	B. gallinarum No. 115	Dr. P. B. Hadley.....	1892	Dr. V. A. Moore
88	B. gallinarum No. 88	Dr. P. B. Hadley.....	1888	Kent, Eng.	Dr. E. Klein
55	From cholera-like epidemic	Dr. Chas. Murray.....	1917	Iowa	Dr. Chas. Murray
47	B. sanguinarium	Apr. '18	Ill.	F. W. Mulsow
56	From cholera-like epidemic	Dr. Chas. Murray.....	Oct. '18	Iowa	Dr. Chas. Murray
57	B. sanguinarium	Nov. '18	Ill.	F. W. Mulsow
40	B. avisepticus No. 549	Am. Mus. Nat. Hist. ..	1908	Colo.	State Vet. Coll.
41	From cholera-like epidemic among wild ducks	Univ. of Kansas.....	1916	Kans.	Dr. B. J. Clawson
42	B. avisepticus "Park"	Univ. of Kansas.....	Dr. W. H. Park
B. avisepticus 44	B. avisepticus	B. A. I., Wash., D. C.	R. I.	Dr. P. B. Hadley
45	B. avisepticus	L. M. Roderick.....	Feb. '18	N. D.	L. M. Roderick
46	B. avisepticus	L. M. Roderick.....	Mar. '18	N. D.	L. M. Roderick
48	B. avisepticus No. 62 (Kral No. 3)	Dr. P. B. Hadley.....	Kral's laboratory
49	B. avisepticus No. 91	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
71	B. avisepticus	Nov. '18	Ill.	F. W. Mulsow
72	B. avisepticus	Oct. '18	Ill.	F. W. Mulsow
B. pullorum 25	B. pullorum No. 102A	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
4	B. pullorum IV	Dr. Theobald Smith...	Dr. Theobald Smith
5	B. pullorum No. 5	Dr. C. M. Haring.....	July '14	Calif.	Dr. C. M. Haring
6	B. pullorum VI	Dr. Theobald Smith...	Dr. Theobald Smith
7	B. pullorum	Dr. G. D. Horton.....	1917	Mo.	Dr. G. D. Horton
8	B. pullorum	Dr. G. D. Horton.....	1917	Mo.	Dr. G. D. Horton
9	B. pullorum No. 9	Dr. C. M. Haring.....	Jan. '17	Calif.	Dr. C. M. Haring
10	B. pullorum	Dr. L. F. Rettger.....	1909	Conn.	Dr. L. F. Rettger
16	B. pullorum	Dr. L. F. Rettger.....	1916	Conn.	Dr. L. F. Rettger
18	B. pullorum "K Rettgers"	Dr. W. A. Hagan.....
19	B. pullorum J2	Dr. W. A. Hagan.....	N. Y.	Dr. F. S. Jones
20	B. pullorum	Dr. W. A. Hagan.....	N. Y.	Dr. F. S. Jones
21	B. pullorum 17	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
22	B. pullorum 34	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
23	B. pullorum 56	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
24	B. pullorum 99	Dr. P. B. Hadley.....	R. I.	Dr. P. B. Hadley
26	B. pullorum F4	Dr. L. F. Rettger.....	1910	Conn.	Dr. L. F. Rettger
27	B. pullorum (W. R.)	Dr. L. F. Rettger.....	1911	Conn.	Dr. L. F. Rettger
28	B. pullorum B	Dr. L. F. Rettger.....	1916	Conn.	Dr. L. F. Rettger
29	B. pullorum W	Dr. L. F. Rettger.....	1917	Conn.	Dr. L. F. Rettger
30	B. pullorum 403	Am. Mus. Nat. Hist. ..	1911	N. Y.	Dr. F. S. Jones
31	B. pullorum	Dr. F. D. Beekwith....	Jan. '18	Oreg.	Dr. F. D. Beekwith
32	B. pullorum	B. A. I., Wash., D. C.	D. C.

DISTRIBUTION

In order to learn more concerning the distribution of these organisms and to secure cultures from as widely different sources as possible, letters were written to every agricultural experiment station in the United States. I wish to express my thanks at this time for cultures and information received from the many correspondents.

From publications and letters from the experiment stations it appears that investigations concerning these organisms have been carried on in only a few states. Hence our knowledge of the diseases caused by these organisms in the United States is quite fragmentary and rests on scattered observations. A few states as Connecticut, Rhode Island and Massachusetts have made quite thoro studies of these organisms and methods of controlling their spread. Their distribution, as obtained from the correspondence and bulletins of the several experiment stations is summarized in table 2.

TABLE 2
DISTRIBUTION OF *B. PULLORUM* AND *B. SANGUINARIUM* BY STATES

No Epidemics Observed	No Studies Made	Occurrence of <i>B. pullorum</i>		Occurrence of <i>B. sanguinarium</i>	
		Rare	Frequent	Rare	Frequent
Ala.	Ariz.	*Iowa	Calif.	Calif.	Conn.
Colo.	Fla.	Kans.	Conn.	Del.	Mass
Mich.	Idaho	Nebr.	*Del.	Iowa	R. I.
Minn.	Ky.	Texas	*Ill.	Mo.	
Miss.	Maine	Wisc.	Ind.	Penn.	
N. J.	Md.	La.	Mass.	Wash.	
N. C.	Mont.		Mo.	Wyo.	
*N. D.	Nev.		N. H.		
*Ohio	N. Mex.		N. Y.		
Okla.	S. C.		Ore.		
*S. D.	Utah		Penn.		
Vt.	Va.		R. I.		
W. Va.					

* Letters from these states contain the information that chicken cholera is rather prevalent.

From table 1 it appears that *B. sanguinarium* has been isolated comparatively few times, but as has been suggested by Taylor¹⁷ and Hadley³ and from my own observations, it appears that this organism is more frequently the cause of epidemics in fowls than it is generally considered to be. Thus, some cultures which I have received from other laboratories that were labelled *B. avisepticus* have proven to be *B. sanguinarium*. Hence many cholera-like epidemics attributed to *B. avisepticus* may very likely be due to *B. sanguinarium*.

¹⁷ Jour. Am. Vet. Med. Assn., 1916, 49, p. 35.

CULTURAL CHARACTERISTICS

Quite thoro descriptions of the staining, morphological, and cultural characteristics on the ordinary mediums of *B. sanguinarium* and *B. pullorum* have been made by Moore⁴ and Rettger,¹⁸ respectively. It seems necessary to mention only a few of their most characteristic reactions. They are gram-negative, nonmotile rods, and belong to the paratyphoid-enteritidis group. *B. sanguinarium* does not, in general, take the ordinary stains as readily as *B. pullorum*, and often when stained with fuchsin the central portion is only faintly stained. The growth on agar and gelatin resembles that of the other members of the paratyphoid-enteritidis group, altho in my work the strains of *B. pullorum* have not grown as a rule as luxuriant on these mediums as *B. sanguinarium* or other members of this group.

INDOL

In Moore's original article it is stated that *B. sanguinarium* produces a slight amount of indol, but other investigators using the more accurate modern methods have not been able to detect indol formation by this organism. *B. pullorum* has never been observed to produce indol.

The test for indol production in the present study has been made in two ways, on three different occasions. The first time the organisms were grown in the standard peptone solution for 1-5 days, respectively. In the second and third tests the organisms were grown in standard meat extract broth and in meat infusion broth, respectively. The test for indol has been made in two ways, with the same results in all cases.

1. By Ehrlich's paradimethyl-amido-benzaldehyde reaction.

2. By the use of vanillin as described by Nelson (J. Biol. Chem., 1916, 24, p. 527).

Control tests were made by testing uninoculated broth, and cultures of *B. Coli*.

Indol was not produced by any strains of *B. pullorum* or *B. sanguinarium*. Seven strains of *B. avisepticus* produced indol, but two strains, 40 and 42, which were labelled *B. avisepticus* did not form indol. These two strains have proven to be similar to strains of *B. sanguinarium* in many other respects; hence, I have called them *B. sanguinarium*. There is some dispute as to the ability of *B. avisepticus* to produce indol. Lignières¹⁹ states that indol is not produced by this organism, while Kitt¹⁹ and others maintain that indol is pro-

¹⁸ Jour. Med. Research, 1908, 18, p. 227.

¹⁹ Handb. d. pathogen, Mikroorganismen, 1913, 6, p. 39.

duced by *B. avisepticus*. My results have been in accord with those of Kitt.

HYDROGEN SULPHID

Since it has been observed by Burnet and Weissenbach,²¹ Jordan and Victorson,²² and others, that certain members of the paratyphoid-enteritidis group differ in their ability to produce hydrogen sulphid, it seemed desirable to test the avian strains in this respect.

The test for hydrogen sulphid production was made by placing 2 drops of a 10% aqueous solution of lead acetate into tubes containing 10 cc of sterile freshly prepared nutrient agar cooled to about 50 C. The tubes were then cooled and stabs were made by passing the inoculating needle between the wall of the tube and the agar. Tests have been made at three different times. The first time meat extract was used in the preparation of the agar and only the strains from 1-20 were tested. In the second, meat infusion broth was used in the preparation of the agar. The third was made according to the method of Jordan and Victorson,²² with the exception that my medium contained Armour's peptone instead of Witte's.

All the strains of *B. pullorum* and *B. sanguinarium*, except *B. pullorum* No. 19 and *B. sanguinarium* No. 3 and No. 14 produced a blackening of the medium along the line of inoculation in 24 hours when tested by the method of Jordan and Victorson. In the second test the *B. sanguinarium* No. 14 was positive in 24 hours and *B. sanguinarium* No. 54 was positive only after 48 hours. These strains which do not form hydrogen sulphid in 24 hours are typical in other respects. On the whole, these avian strains are like *B. enteritidis* and *B. paratyphoid B* in regular hydrogen sulphid production.

MILK

It has been stated by Smith and Ten Broeck,¹ Rettger and Koser,²³ and others that *B. sanguinarium* produces an alkaline reaction in milk in a few days, while *B. pullorum* remains acid. Their observations extended over a period of about two weeks.

In the present study I used certified milk from which the cream had been removed. The milk was sterilized by heating in the autoclave for 15-20 minutes at 10 lbs. of pressure. Either 7% of a 1% solution of Merck's litmus or 1% of a 0.5% solution of brom-cresol-purple prepared as recommended by Clark and Lubs, was added to the milk before sterilization. The two indicators have given practically the same results altho the brom-cresol-purple appears to be the more delicate and is not so easily reduced.

²¹ Compt. rend. Soc. de biol., 1915, 78, p. 565.

²² Jour. Infect. Dis., 1917, 21, p. 571.

²³ Jour. Med. Research, 1917, 35, p. 443.

All strains produce a slight acidity in 24-48 hours. The strains of *B. sanguinarium* from alkali, however, after 2-5 days. There are some exceptions to this which are shown in table 3. All strains of *B. pullorum* produce alkali after 60-days' incubation. Some strains showed an alkaline reaction as early as 4 days, while others did not become alkaline until about 60 days. The reaction varies considerably with different lots of milk.

Since certain strains of *B. pullorum* form alkali as early as some strains of *B. sanguinarium* it appears that we cannot differentiate between these two organisms by any change produced in the reaction of milk. Altho, in general, *B. sanguinarium* produces alkali earlier than *B. pullorum*. The *B. avisepticus* produces a slight acidity in 48 hours which persists for 90 days, but the organisms are usually dead after about 2 weeks' incubation. Thus it may be distinguished from the other organisms by its action on milk.

The digestive action of these organisms on the casein was tested with milk which contained no indicator. *B. sanguinarium*, in general, completely digested the casein in about 2 weeks, altho with some strains digestion was not complete before 6 weeks. The strains of *B. pullorum* vary even more in their digestive action; with some strains digestion was complete in 2 weeks, while others have failed in this respect in 90 days. Therefore these organisms cannot be differentiated by their digestive action on milk.

Details of the changes in the reaction of milk produced by these organisms are given in table 3.

FERMENTATION OF CARBOHYDRATES

The fermentation reactions of *B. sanguinarium* and *B. pullorum* have been studied quite extensively by Rettger and Koser,²³ and more recently by Krumwiede and Kohn.²⁴ The former investigators state that the principal fermentative differences between these organisms are: (1) The production of gas by *B. pullorum* in dextrose, levulose, galactose, and mannite, and the inability of *B. sanguinarium* to form gas in any of the carbohydrates, and (2) the production of acid in maltose, dulcitol, and dextrin by *B. sanguinarium* and the failure of *B. pullorum* to ferment these substances. Similar reactions have been observed by Goldberg²⁵ and others.

The ability of *B. pullorum* to form gas in some carbohydrates seems to vary with different strains and at times the same strain will show variations in its ability to produce gas. Rettger isolated a strain which did not form gas in any of the carbohydrates. He has also observed variations in gas pro-

²⁴ Ibid., 1917, 36, p. 509.

²⁵ Jour. Am. Vet. Med. Assn., 1917, 51, p. 203.

duction with other strains. Variation in the ability to form gas has also been noted by Krumwiede and Kohn,²⁴ who secured a non-gas producing strain from Smith, but in their work this strain was observed to produce gas.

TABLE 3
CHANGES PRODUCED IN THE ACIDITY OF MILK CONTAINING BROM-CRESOL-PURPLE,
BY DAYS

	1	2	3	4	5	7	10	14	21	30	60	90
<i>B. sanguinarum</i>	1	+	+c	—
	2	+	+c	—
	3	+	+c	—
	11	+c	—
	12	+c	Nc—	—
	13	+	+c—	—
	14	+c	Nc—	—
	15	+	+c—	Nc—	—
	40	+c	Nc—	—
	41	+c	—
	42	+c	—
	47	+c	Nc—	—
	50	+	+	—	..
	51	+c	—
	52	+	..	+c—	..	Nc—	—
	53	+	+	—	..
	54	+c	—
	55	+c	—
	88	+	+c	—
	56	+	..	Nc—	—
	57	+	—
<i>B. avisepticus</i>	44	+	+	+	+	+
	45	+	+	+	+	+
	46	+	+	+	+	+
	48	+	+	+	+	+
	49	+	+	+	+	+
	71	+	+	+
	72	+	+	+
<i>B. pullorum</i>	4	+	+c	—
	5	+	+c	—
	6	+	+	—	..
	7	+	+	—	..
	8	+	+	—	..
	9	+	—
	10	+	+	—	..
	16	+	—
	18	+	—
	19	+	+c	—
	20	+c	—
	21	+	—
	22	+	—
	23	+	+c—	..	—
	24	+c	—
	25	+	—
	26	+	—
	27	+	+c	Nc—	—
	28	+	..	+c—	..	Nc—	—
	29	+	+	—	..
	30	+	—
	31	+	N	—	..
	32	+	+c—	—

Symbols: + = acid.

+c = body of tube acid and cream ring alkaline.

Nc = body of tube neutral and cream ring alkaline.

— = alkaline reaction thruout medium.

In my own work the medium used in determining the fermentative reactions has been standard nutrient broth made from meat extract to which was added 0.5% of the carbohydrate to be tested. Andrade's indicator was used as the indicator of acid production in some of the earlier tests but brom-cresol-purple has been used in all the later tests. The indicator was added

to the medium which was then placed in tubes containing inverted vials. The medium was sterilized by heating in the autoclave for 10-15 minutes at 10 lbs. of pressure.

Some preliminary tests were made in which the amount of acid produced was determined by titration. But since this required considerable amounts of the medium in order to make observations at intervals over a period of several days and gave no information of importance other than was given by the indicator method, it has been discontinued.

The fermentative reactions are summarized in tables 4 and 5.

TABLE 4
ACID AND GAS PRODUCTION IN THE CARBOHYDRATES BY *B. PULLORUM*

Acid and Gas Positive	Acid and Gas Negative		Gas Variable and Acid Positive
Dextrose	Lactose	Erythrit	Xylose
Levulose	Saccharose	Dextrin	Sorbitol
Galactose	*Maltose	Dulcitol	
Mannitol	Raffinose	Inositol	
Mannose	Inulin	Salicin	
Arabinose			
Rhamnose			

* Some strains of *B. pullorum* apparently ferment maltose, and *B. avisepticus* produces a very slight amount of acid sometimes.

TABLE 5
ACID PRODUCTION IN THE CARBOHYDRATES BY *B. SANGUINARIUM* AND *B. AVISEPTICUS*

<i>B. sanguinarium</i>		<i>B. avisepticus</i>	
Positive	Negative	Positive	Negative
Dextrose	Lactose	Dextrose	Lactose
Levulose	Saccharose	Levulose	Rhamnose
Galactose	Raffinose	Galactose	†Maltose
Mannitol	Inulin	Mannitol	
Mannose	Erythrit	Mannose	Dulcitol
Arabinose	Inositol	Arabinose	
Rhamnose	Salicin		
Dulcitol	†Sorbitol	Sorbitol	
Maltose		Saccharose	
Xylose		Xylose	
*Dextrin			

* Only a very slight amount of acid was formed by some strains, which made it practically impossible to distinguish from *B. pullorum* by the use of this carbohydrate alone.

† Acid production in sorbitol varies with different strains as will be shown in a subsequent table.

‡ Some strains of *B. pullorum* apparently ferment maltose, and *B. avisepticus* produces a very slight amount of acid sometimes.

The fermentative reactions, however, are not as uniform as tables 4 and 5 indicate. In some cases it is rather difficult to differentiate strains of *B. pullorum* from *B. sanguinarium* by their fermentative capacities.

It is generally considered that maltose fermentation differentiates between *B. pullorum* and *B. sanguinarium*. Altho Krumwiede and

Kohn have observed that certain strains of *B. pullorum* produce a slight amount of acid in maltose after several days' incubation, they suggest that this is probably due to hydrolysis rather than any latent ability to ferment this carbohydrate. In my own work some strains of *B. pullorum* have produced a slight amount of acid after several days' incubation. This does not appear to be due to hydrolysis alone, since those strains which produce acid after several days' incubation do not form acid sooner in tubes which have been incubated for 3 weeks than in those freshly made up. Strains 20 and 32 have produced acid and gas in maltose somewhat irregularly. Subcultures of colonies picked from plates of these two strains are variable in their fermentation of maltose. In other respects these strains are typical. There also appears to be some variation in the fermentation of maltose by *B. sanguinarium*. Two strains have not fermented maltose promptly. They have fermented it only slightly after 10 days' incubation. The reaction in maltose and a few other carbohydrates, in which the reactions are quite variable, are given in table 6.

Dulcite is not fermented by any strain of *B. pullorum*, while all strains of *B. sanguinarium* but one ferment it promptly; Strain 14 ferments it rather slowly. There appears to be less variation in the fermentative action of these avian strains on dulcite than in any other carbohydrate.

Dextrin is fermented only slightly by most strains of *B. sanguinarium* and is not fermented at all by certain strains at different times. On the other hand, some strains of *B. pullorum* appear to ferment this carbohydrate slightly. Krumwiede and Kohn²⁴ have also observed that the fermentation of dextrin by *B. sanguinarium* is irregular. They believe that the results depend on the constituents of the sample, since other members of the paratyphoid-enteritidis group varied in a similar manner.

Acid is uniformly, tho slowly, produced in sorbite by *B. pullorum*, but the ability to form gas varies. *B. sanguinarium* is irregular in its action on sorbite. Some strains do not produce acid in 30 days, and in no case is acid formed before 5-7 days. The strains of *B. avisepticus* ferment this carbohydrate promptly.

Rhamnose is promptly fermented by all strains of *B. pullorum*, altho the gas production is somewhat irregular. The inability of *B. sanguinarium* to form acid promptly in rhamnose is quite uniform with all strains and may serve in a measure to differentiate between

B. sanguinarium and *B. pullorum*. The strains of *B. avisepticus* have not fermented this carbohydrate.

The chief fermentative differences between *B. pullorum* and *B. sanguinarium* are:

1. The production of gas by most strains of *B. pullorum* in several of the carbohydrates and the inability of *B. sanguinarium* to produce gas in any carbohydrate.

2. The fermentation of dulcitol and maltose by *B. sanguinarium* and the inability of *B. pullorum* to ferment these carbohydrates. A few strains of *B. pullorum* attack maltose slightly and two strains of *B. sanguinarium* produce acid very slowly in this sugar. Also Strains 20 and 32 of *B. pullorum* irregularly produce acid and gas in maltose.

3. The strains of *B. sanguinarium* studied ferment rhamnose slowly while the strains of *B. pullorum* ferment it promptly.

4. Some strains of *B. sanguinarium* do not produce acid in sorbitol, while all strains of *B. pullorum* produce acid.

AGGLUTINATION

The agglutinative relationship of these organisms to each other and to *B. typhosus* was first observed by Smith and Ten Broeck.¹ They also observed that these organisms were not agglutinated by serums of rabbits immunized toward the different types of *B. dysenteriae*, and to *B. cholerae* suis. Rettger and Koser²³ and Krumwiede and Kohn have also observed the close agglutinative relationship of *B. sanguinarium* and *B. pullorum* to *B. typhosus*. Pfeiler and Roepke⁷ have observed that an immune serum for the fowl typhoid organism did not agglutinate *B. typhosus* or *B. suispestifer* but agglutinated *B. paratyphosus* A and B, *B. enteritidis* and *B. psittacosis* in dilutions as high as 1:400.

In my work all determinations have been made by mixing 1 cc of the various dilutions of the serum in small tubes, with 1 cc of a 24-hour agar slant growth suspended in sufficient salt solution to give a uniform turbidity. The mixtures were incubated for 2 hours at 37 C., then placed in the ice chest for about 20 hours, when readings were made.

Agglutination tests with serum of rabbits immunized toward Strains 4, 7, 9, 19, 22 and 31 of *B. pullorum*, and Strains 2, 3, 11, 40 and 47 of *B. sanguinarium* have not shown any agglutinative differences between *B. pullorum* and *B. sanguinarium*. The strains with which I have worked have not been agglutinated when treated with a 1:250 dilution of serums from rabbits immunized toward typical strains of *B. paratyphosus* A and B, *B. suispestifer*, *B. abortus equinus*, and the Shiga and Flexner types of *B. dysenteriae*.

The agglutinative relation to *B. typhosus*, which has been observed by other investigators, has been observed in my own work. The

immune serum for *B. typhosus* 189—a stock culture—has not, as a rule, agglutinated the avian strains in as high dilutions as has that for *B. typhosus* “Hopkins,” when the titer of the serums was the same. All strains of *B. pullorum* and *B. sanguinarium* have been tested with these two serums. Some typical reactions are given in table 7.

TABLE 6
ACID AND GAS PRODUCTION IN BROTH CONTAINING 0.5% OF THE CARBOHYDRATE AND 1% OF A 0.5% SOLUTION OF BROM-CRESOL-PURPLE

Strain	Dextrose		Maltose		Arabinose		Rhamnose		Sorbitol		Xylose		Dulcitol	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
<i>B. sanguinarium</i> 1	+	—	+	—	+	—	+3	—	+10	—	+	—	+	—
2	+	—	+	—	+	—	+3	—	+7	—	+	—	+	—
3	+	—	+	—	+	—	+2	—	+10	—	+	—	+	—
11	+	—	+3	—	+	—	+3	—	+10	—	+	—	+	—
12	+	—	+	—	+	—	+3	—	+14	—	+2	—	+	—
13	+	—	+	—	+	—	+2	—	+10	—	+2	—	+	—
14	+	—	+21	—	+	—	+2	—	+21	—	+	—	+14	—
15	+	—	+	—	+	—	+2	—	+21	—	+	—	+	—
40	+	—	+	—	+	—	+3	—	—	—	+	—	+	—
41	+	—	+	—	+	—	+3	—	+21	—	+	—	+	—
42	+	—	+	—	+	—	+2	—	+21	—	+	—	+	—
47	+	—	+	—	+	—	+3	—	+14	—	+	—	+	—
50	+	—	+14	—	+	—	+5	—	—	—	+	—	+	—
51	+	—	+	—	+	—	+3	—	—	—	+	—	+	—
52	+	—	+	—	+	—	+5	—	+10	—	+	—	+	—
53	+	—	+	—	+	—	+7	—	+21	—	+	—	+	—
54	+	—	+	—	+	—	+5	—	+21	—	+	—	+	—
55	+	—	+	—	+	—	+10	—	—	—	+	—	+	—
88	+	—	+	—	+	—	+5	—	+21	—	+	—	+	—
<i>B. pullorum</i> 4	+	+	+30	—	+	+	+	+	+6	+7	+	+2	—	—
5	+	+	+21	—	+	+2	+	+	+7	+14	+	+2	—	—
6	+	+	—	—	+	—	+	+2	+10	—	+	—	—	—
7	+	+	—	—	+	+	+	+2	+10	—	+	—	—	—
8	+	—	—	—	+2	—	+	—	+7	—	+	—	—	—
9	+	+	—	—	+	+	+	+2	+10	—	+	+6	—	—
10	+	+2	±30	—	+	—	+	+4	+10	—	+6	—	—	—
16	+	+2	—	—	+2	+2	+	—	+7	—	+6	—	—	—
17	+	+	+10	—	+	+	+	+	+10	—	+	+2	—	—
19	+	+	+30	—	+	+	+	—	+7	+7	+	+2	—	—
20	+	+	+21	±21	+	+2	+	+	+7	+7	+	+2	—	—
21	+	+	—	—	+	+3	+	+	+6	—	+	—	—	—
22	+	+	+30	—	+	—	+	+2	+6	+14	+2	+6	—	—
23	+	+	—	—	+	—	+	+	+10	—	+	—	—	—
24	+	+	—	—	+	+2	+	+	+10	+14	+	+3	—	—
25	+	+	—	—	+	—	+	+2	+10	—	+	—	—	—
26	+	+	+30	—	+2	—	+	+3	+10	—	+	+3	—	—
27	+	+	—	—	+	+	+	+	+7	—	+	+2	—	—
28	+	+2	—	—	+	+2	+	+3	+10	+30	+3	—	—	—
29	+	+	+30	—	+	+2	+	+2	+10	—	+2	—	—	—
30	+	+2	—	—	+	+	+	+2	+10	—	+2	—	—	—
31	+	+	—	—	+	+	+	+	+10	+10	+	+6	—	—
32	+	+	+3	+3	+	+	+	+	+10	+10	+	+3	—	—
<i>B. avisepticus</i> 44	+	—	±	—	+	—	—	—	+2	—	+5	—	—	—
45	+	—	±	—	+	—	—	—	+2	—	+7	—	—	—
46	+	—	±	—	+	—	—	—	+2	—	+5	—	—	—
48	+	—	±	—	+	—	—	—	+	—	±7	—	—	—
49	+	—	±	—	+	—	—	—	+2	—	±7	—	—	—

Symbols: + = acid or gas production in 24 hours.

— = no acid or gas production in 30 days.

A figure following a (+) indicates the number of days after inoculation when acid or gas was first observed.

± = very slight acid production or irregularity in acid production.

TABLE 7

AGGLUTINATION OF *B. PULLORUM* AND *B. SANGUINARIUM* WITH SERUMS FROM RABBITS
IMMUNIZED TO *B. TYPHOSUS*

	Serum of Rabbit Treated with <i>B. typhosus</i> Hopkins Titer 1:5000				Serum of Rabbit Treated with <i>B. typhosus</i> 189 Titer 1:5000			
	Dilution of Serum				Dilution of Serum			
	500	1000	2500	5000	500	1000	2500	5000
<i>B. sanguinarium</i> 1.....	++	+	+	—	++	+	—	—
11.....	+++	++	++	+	++	++	+	—
14.....	++	++	+	—	++	++	+	—
47.....	+++	+++	++	+	+++	++	++	+
<i>B. pullorum</i> 9.....	+++	+++	+	+	++	+	—	—
16.....	+++	+	+	—	++	+	+	—
22.....	+++	+++	++	+	+++	++	+	+
31.....	+++	++	+	—	++	++	+	—

Symbols: +++ = complete agglutination.
 ++ = marked agglutination.
 + = slight agglutination.
 — = no agglutination.

The agglutinative relation between *B. enteritidis* and these organisms has not been reported by other investigators, except that Pfeiler and Roepke observed that immune serums for two strains of *B. enteritidis* with a titer of 1:10,000 and 1:16,000, respectively, agglutinated the "bacillus of fowl typhoid" in a dilution as high as 1:800. In my work, immune serums toward two strains of *B. enteritidis* (Jordan's strains 52 and 205) have agglutinated all the avian strains. Some strains of *B. pullorum* and *B. sanguinarium*, however, have been only slightly agglutinated in dilutions of 1:250 when the titer was 1:10,000. In general, the serum of the rabbit immunized to *B. enteritidis* (52) has agglutinated the avian strains in higher dilutions than the immune serum for *B. enteritidis* (205). Some typical reactions are given in table 8.

TABLE 8

AGGLUTINATION OF *B. PULLORUM* AND *B. SANGUINARIUM* WITH SERUMS FROM RABBITS
IMMUNE TO *B. ENTERITIDIS*

	Serum of Rabbit Treated with <i>B. enteritidis</i> 52 Titer 1:16,000				Serum of Rabbit Treated with <i>B. enteritidis</i> 205 Titer 1:16,000			
	Dilution of Serum				Dilution of Serum			
	100	250	500	1000	100	250	500	1000
<i>B. sanguinarium</i> 2.....	+++	++	+	—	+++	++	—	—
3.....	+++	++	+	+	+++	++	+	—
11.....	+++	++	+	—	+++	+	+	—
47.....	+++	+++	++	++	+++	++	++	+
50.....	+++	++	++	+	+++	++	—	—
52.....	++	+	+	—	+++	+	+	—
<i>B. pullorum</i> 4.....	+++	++	+	—	+++	++	+	—
5.....	+++	+++	++	+	+++	++	++	+
7.....	+++	++	+	—	++	++	+	—
20.....	++	+	—	—	+++	++	+	—
22.....	+++	++	++	+	+++	+	+	+
32.....	+++	++	++	+	+++	++	+	—

Symbols are the same as those in Table 7.

The agglutinative powers of serums of rabbits immunized toward several avian strains have been tested. The serums of rabbits, immunized toward Strains 4, 7, 9, 19, 22 and 31 of *B. pullorum*, and Strains 2, 3, 11, 40 and 47 of *B. sanguinarium* have in general reacted alike in all respects. These serums have not agglutinated in 1:500 dilution typical strains of *B. paratyphosus* A and B, *B. suispestifer*, *B. dysenteriae* Flexner, *B. dysenteriae* Shiga, *B. coli*, *B. proteus vulgaris*, and *B. avisepticus*. Strains of *B. typhosus*, *B. enteritidis*, and *B. abortus equinus*, however, have been agglutinated by immune *B. pullorum* or *B. sanguinarium* sera.

Nine strains of *B. enteritidis* have been tested with several of these immune serums. A few strains of *B. enteritidis* have been agglutinated in as high dilutions as some of the avian strains. It thus appears that *B. enteritidis* is agglutinated in higher dilutions by these sera, than *B. sanguinarium* or *B. pullorum* is agglutinated by immune *B. enteritidis* serum when the titer of the serums is the same. The agglutinative action, on *B. enteritidis*, of the serum of a rabbit immunized to *B. pullorum*, is given in table 9.

TABLE 9
AGGLUTINATION OF *B. ENTERITIDIS* WITH IMMUNE SERUM TO *B. PULLORUM* (9).
TITER 1:10,000

	1:500	1:1000	1:2500	1:5000	1:10,000
<i>B. sanguinarium</i> 3.....	+++	+++	++	+	—
<i>B. pullorum</i> 9.....	+++	+++	++	++	+
<i>B. enteritidis</i> 50.....	+++	++	+	—	—
51.....	+++	++	+	—	—
52.....	+++	++	+	—	—
53.....	+++	++	+	—	—
153.....	+++	+++	++	+	—
204.....	+++	++	++	—	—
205.....	+++	++	—	—	—
207.....	+++	++	+	—	—
228.....	+++	++	+	—	—

The agglutination of *B. typhosus* with serums of rabbits immunized to *B. sanguinarium* or *B. pullorum* was first observed by Smith and Ten Broeck. Their results have since been confirmed by Rettger and Koser, and Krumwiede and Kohn. Pfeiler and Roepke, however, have observed that a serum, with a titer of 8,000, from a rabbit immunized toward the "bacillus of fowl typhoid" did not agglutinate *B. typhosus* in a dilution of 1:100. In the present study three strains of *B. typhosus* have been agglutinated in high dilutions with serums of rabbits immune to the avian strains. The stock culture *B. typhosus*

189 generally did not agglutinate as well as the other two strains. Typical results are given in table 10.

TABLE 10
AGGLUTINATION REACTION OF *B. PULLORUM* (19) SERUM. TITER 1:5000

Strain	Dilution of Serum						
	100	250	500	1,000	2,500	5,000	10,000
<i>B. sanguinarium</i> 1.....	+++	++	+	—	—
41.....	+++	+	+	—	—
47.....	+++	+++	++	+	—
<i>B. pullorum</i> 10.....	+++	+++	++	+	—
22.....	+++	+++	++	+	+
31.....	+++	+++	+	—	—
<i>B. typhosus</i> Hopkins.....	+++	++	+	—	—
189.....	++	++	—	—	—
197.....	+++	++	++	—	—
<i>B. enteritidis</i> 52.....	+++	+++	+	+	—
205.....	+++	++	+	—	—
228.....	++	+	—	—	—
<i>B. abortus equinus</i> 192.....	+++	++	+	—	—
193.....	+++	+++	++	—	—
196.....	+++	+++	++	+	—
<i>B. avisepticus</i> 44.....	+	—	—	—	—	—	—
46.....	—	—	—	—	—	—	—
48.....	—	—	—	—	—	—	—
<i>B. paratyphosus</i> A 4.....	—	—	—	—	—	—	—
<i>B. paratyphosus</i> B 12.....	+	—	—	—	—	—	—
<i>B. suispestifer</i> 118.....	—	—	—	—	—	—	—

TABLE 11
AGGLUTINATION REACTION OF *B. SANGUINARIUM* (40) SERUM. TITER 1:10,000

Strain	Dilution of Serum						
	100	250	500	1,000	2,500	5,000	10,000
<i>B. sanguinarium</i> 2.....	+++	+++	+++	++	+
14.....	+++	++	+	—	—
88.....	+++	+++	++	+	—
<i>B. pullorum</i> 8.....	+++	+++	++	+	—
22.....	+++	+++	++	++	+
31.....	+++	++	++	+	—
<i>B. typhosus</i> Hopkins.....	+++	+++	++	+	—
189.....	+++	+++	++	—	—
197.....	+++	+++	++	+	—
<i>B. enteritidis</i> 52.....	+++	++	+	—	—
153.....	+++	++	++	+	—
206.....	+++	++	—	—	—
<i>B. abortus equinus</i> 192.....	+++	++	++	+	—
195.....	+++	++	++	+	—
196.....	+++	+++	++	+	+
<i>B. avisepticus</i> 44.....	—	—	—	—	—	—	—
45.....	—	—	—	—	—	—	—
49.....	—	—	—	—	—	—	—
<i>B. paratyphosus</i> A 4.....	—	—	—	—	—	—	—
<i>B. paratyphosus</i> B 12.....	—	—	—	—	—	—	—
<i>B. suispestifer</i> 118.....	—	—	—	—	—	—	—

In studying the agglutinative relation of the avian strains to *B. abortus-equinus* it was observed that the serums of rabbits immunized to *B. pullorum* or *B. sanguinarium* contained agglutinins for five strains of *B. abortus-equinus*. Rettger and Koser, however, observed that a strain of *B. abortus* used as a control was not agglutinated by

immune *B. pullorum* serum. In my work it was noted that *B. pullorum* and *B. sanguinarium* were not agglutinated by the serum of a rabbit immunized toward *B. abortus-equinus*.

Some typical agglutinative reactions of serums from rabbits immunized to *B. pullorum* are given in Table 10.

Results similar to those in the preceding tables have been observed with serums of rabbits immunized toward other strains of *B. sanguinarium* and *B. pullorum*, but it does not seem necessary to give tables. The agglutination of *B. typhosus*, *B. enteritidis*, and *B. abortus-equinus* with such serums is a rather remarkable case of cross agglutination. These results suggest that *B. enteritidis* is more clearly related, at least antigenically, to these organisms and to *B. typhosus* than other members of the paratyphoid-enteritidis group.

ABSORPTION TESTS

In order to test further the antigenic relation of these organisms to *B. typhosus*, *B. enteritidis*, and *B. abortus-equinus*, absorption tests were made. The tests were carried out by suspending in about 25 cc of isotonic salt solution, the 24-hour growth on an agar slant, whose surface area was approximately 60 sq. cm. The suspension was centrifugalized and the supernatant liquid poured off. In some tests the organisms were washed a second time but this did not change the results. The organisms were then suspended in 5 cc of salt solution and to this suspension was added 5 cc of a 1:25 dilution of the serum to be tested. This mixture was placed in a small flask and incubated for 3 hours at 37 C. and then placed in the ice box for 20-48 hours. They were then centrifugalized and the remaining titer for the various organisms determined.

TABLE 12
ABSORPTION TESTS WITH SERUM OF RABBIT TREATED WITH *B. TYPHOSUS* 189,
TITER 1:20,000

Exhausted with	Original Titer	Remaining Titer for		
		<i>B. typhosus</i> Hopkins	<i>B. pullor-</i> um 22	<i>B. sanguin-</i> arium 47
<i>B. typhosus</i> 189.....	20,000	<100	<100	<100
<i>B. sanguinarium</i> 47.....	20,000	10,000	<100	250
<i>B. pullorum</i> 22.....	20,000	20,000	100	250
<i>B. enteritidis</i> 52.....	100	20,000	<100	100

In absorption tests with typhoid immune serum it was observed that *B. sanguinarium* or *B. pullorum* removed the agglutinins for these avian strains but removed only a small percentage or none of the agglutinins for *B. typhosus*. Smith and Ten Broeck obtained similar results. *B. enteritidis* also removed a large percentage or all of the agglutinins for the avian strains. Typical results are given in table 12.

With the serums of rabbits immunized toward *B. enteritidis*, it was observed that the avian strains did not remove the agglutinins for *B. enteritidis*. The three strains of *B. typhosus*, which I have used, removed the agglutinins for the avian strains from these sera. table 13 shows typical reactions.

TABLE 13
ABSORPTION TESTS WITH SERUM OF RABBIT TREATED WITH *B. ENTERITIDIS* (52)

Exhausted with	Original Titer	Remaining Titer for		
		<i>B. enteritidis</i> 52	<i>B. sanguinarium</i> 47	<i>B. pullorum</i> 22
<i>B. enteritidis</i> 52.....	10,000	100	—	—
<i>B. sanguinarium</i> 47.....	2,500	10,000	—	—
<i>B. pullorum</i> 22.....	2,500	10,000	—	—
<i>B. typhosus</i> Hopkins.....	<100	10,000	100	—

From the results indicated in the preceding tables it appears that *B. typhosus* and *B. enteritidis* have a certain antigenic relation to each other which is common to the avian strains. Thus the agglutinins for the avian strains, which have been studied in this respect, are removed from typhoid immune serum by *B. enteritidis*, and from immune *B. enteritidis* serum by *B. typhosus*. Further antigenic relations between *B. typhosus* and *B. enteritidis* are shown in absorption tests with serums of rabbits immunized toward *B. pullorum* or *B. sanguinarium*.

Absorption tests with serums of rabbits immunized toward *B. pullorum* (9) and (22) and *B. sanguinarium* (40) are given in table 14, 15 and 16.

Results similar to those tabulated in these tables have been obtained with serums of rabbits immunized toward *B. pullorum* 19 and 31, and toward *B. sanguinarium* 47 and 11. According to the tables the strains of *B. typhosus* and *B. enteritidis* remove a small percentage of the agglutinins for strains of *B. pullorum* or *B. sanguinarium* from serums of rabbits immunized with some strains of *B. pullorum* or *B. sanguinarium*. Smith and Ten Broeck observed that their strains of *B. typhosus* removed the agglutinins for the avian strains from serums of rabbits immunized toward *B. sanguinarium* or *B. pullorum*. I also noted in some earlier tests that *B. typhosus* or *B. enteritidis* irregularly removed all the agglutinins from such serums. In the earlier tests the organisms were not washed, and it was observed that those growths on slant agar surfaces, which had the most water of con-

densation, removed a large percentage or all the agglutinins. The results were quite irregular with unwashed organisms, but with washed organisms the results were much more regular. The agar on which the organisms were grown had a reaction of +1 to phenolphthalein.

TABLE 14
ABSORPTION TESTS WITH SERUM OF RABBIT TREATED WITH *B. PULLORUM* (9)

Exhausted with	Original Titer	Remaining Titer for			
		<i>B. pullorum</i> 22	<i>B. typhosus</i> Hopkins	<i>B. enteritidis</i> 52	<i>B. abortus</i> equinus 196
<i>B. pullorum</i> 9.....	5,000	100	100	100	100
<i>B. typhosus</i> Hopkins....	5,000	1,600	100	800	200
<i>B. enteritidis</i> 52.....	2,500	2,500	2,500	100	800
<i>B. abortus</i> equinus 196...	2,500	5,000	2,500	800	200

TABLE 15
ABSORPTION TESTS WITH SERUM OF RABBIT TREATED WITH *B. PULLORUM* (22)

Exhausted with	Original Titer	Remaining Titer for				
		<i>B. sanguinarium</i> 47	<i>B. pullorum</i> 22	<i>B. typhosus</i> Hopkins	<i>B. enteritidis</i> 52	<i>B. abortus</i> equinus 196
<i>B. pullorum</i> 9.....	5,000	250	500	250	250	<100
<i>B. sanguinarium</i> 3.....	10,000	100	100	<100	<100	<100
<i>B. typhosus</i> Hopkins.....	5,000	5,000	2,500	100	2,500	5,000
<i>B. enteritidis</i> 52.....	4,000	10,000	5,000	1,000	100	2,500
<i>B. abortus</i> equinus 196.....	5,000	10,000	5,000	5,000	2,500	250

TABLE 16
ABSORPTION TESTS WITH SERUM OF RABBIT TREATED WITH *B. SANGUINARIUM* (40)

Exhausted with	Original Titer	Remaining Titer for				
		<i>B. sanguinarium</i> 47	<i>B. pullorum</i> 22	<i>B. typhosus</i> Hopkins	<i>B. enteritidis</i> 52	<i>B. abortus</i> equinus 196
<i>B. pullorum</i> 22.....	10,000	100	100	<100	100	<100
<i>B. sanguinarium</i> 47.....	10,000	<100	<100	<100	<100	<100
<i>B. typhosus</i> Hopkins.....	5,000	10,000	5,000	100	1,000	1,000
<i>B. enteritidis</i> 52.....	5,000	10,000	5,000	1,000	250	1,000
<i>B. abortus</i> equinus 196.....	5,000	10,000	5,000	2,500	2,500	100

B. typhosus apparently removed a large percentage of the agglutinins from such serums for *B. enteritidis*, and on the other hand, *B. enteritidis* appeared to remove a large percentage of the agglutinins from these serums for *B. typhosus*. *B. abortus*-equinus did not remove agglutinins for other organisms to any great extent.

While absorption tests are not necessary to differentiate *B. abortus equinus* or *B. enteritidis* from these avian strains, yet it was thought that these might throw some light on the antigenic properties of the avian types. From absorption tests as well as from agglutination tests it appears that *B. enteritidis* occupies an intermediate position between *B. typhosus* and *B. abortus-equinus* in its relation to the avian strains. These absorption tests also indicate an antigenic relation between *B. typhosus* and *B. enteritidis*.

PATHOGENESIS

A high percentage of fatal infections following the feeding of *B. pullorum* to rabbits, guinea-pigs, and cats has been reported by Rettger, Hull and Sturges. Smith and Ten Broeck have observed rapidly fatal results following the intravenous injection of broth cultures of *B. sanguinarium* into rabbits. The latter investigators considered the sudden onset of symptoms to be due to a toxin, since if animals recovered from the immediate effects they died a few days later from an infection. In the present study, pathogenesis has been tested by feeding and intraperitoneal inoculations.

FEEDING EXPERIMENTS

In the feeding experiments the cultures have been grown on agar slants, in milk and in meat infusion broth. The broth and milk cultures were incubated from 1-5 days, before they were fed to the animals. The longer incubation period was used to determine the development of toxic substances which might show their presence by producing symptoms of food poisoning when ingested. The method of administering the test organisms varied. In some instances especially in feeding rabbits and guinea-pigs large amounts, water suspensions of 24-hour slant agar cultures were mixed with finely ground carrots, while at other times these suspensions or broth cultures were mixed with milk. In some experiments with cats, rats, and mice finely ground meat was employed which had been sterilized and inoculated with the organisms to be tested and incubated for 24-48 hours. In feeding the birds the suspensions of the cultures were added to dried bread crumbs. The amounts of cultures fed varied from 1-12 agar slant cultures, or in the case of broth or milk cultures from 5-30 c.c. In some cases, where counts were made by plating on agar, approximately 100 billion bacteria were fed.

Different strains of *B. pullorum* have been fed to 7 guinea-pigs weighing between 200 and 350 gm.; 6 rabbits weighing between 1,500 and 2,500 gm.; 2 young rabbits weighing about 700 gm.; 5 kittens weighing between 620 and 925 gm.; 6 mice; 4 rats; 2 cats; 6 sparrows; 5 young pigeons less than 48 hours of age; 2 adult pigeons; 10 chicks about 2 weeks of age, and 4 young hens about 4 months old. Cultures of *B. sanguinarium* have been fed in a similar manner to about the same number of animals.

No symptoms of disease were observed following the ingestion of these organisms, except that one guinea-pig, which was fed 15 c c of a 48-hour milk culture of *B. pullorum* (9), appeared sick 4 days after it was fed the culture and died the following day. No lesions, other than a slight congestion of the abdominal viscera, were observed at necropsy. *B. pullorum* was isolated in pure culture from the heart blood and liver.

Cats, which were fed sparrows, or rabbits that had died from an infection with *B. pullorum* or *B. sanguinarium*, showed no symptoms of disease. Also the freshly isolated strains, *B. pullorum* (31) fed within 2 weeks after isolation from a dead chick, and *B. sanguinarium* (47 and 57) fed 4-7 days after isolation, did not produce any injurious effect when fed to rabbits, guinea-pigs, mice, young and adult pigeons and sparrows. Chickens apparently are not very susceptible to laboratory cultures of these avian strains.

Rettger and others consider that chickens more than 5 days old are generally not readily susceptible to *B. pullorum*. Jones,¹² Rettger,² and Hadley,¹³ however, have observed fatal infections in adult fowls with this organism. Pfeiler and Roepke⁷ working with *B. sanguinarium* have observed that chickens cannot regularly be infected by feeding cultures of this organism, but feeding the viscera of fowls dead of the disease generally produces the disease. They also observed that turkeys, guineas, and peacocks contracted the disease under natural conditions, but ducks and geese did not. They succeeded, however, in producing a fatal infection in a duck by inoculation. One of the strains of *B. sanguinarium* (41) which I have studied was isolated from a wild duck, which with several hundred others had apparently died from an infection with this organism.

From the feeding experiments it appears that rabbits, guinea-pigs, cats, rats, mice, sparrows, pigeons and chickens are not susceptible to laboratory cultures of *B. pullorum* or *B. sanguinarium* when administered by mouth.

INOCULATION EXPERIMENTS

Inoculations of cultures of these organisms into laboratory animals have not given uniform results with the different investigators. Smith and Ten Broeck observed that 0.3-0.5 c c of a 24-hour broth culture of *B. sanguinarium* produced a fatal infection in rabbits when injected intravenously. Taylor,¹⁷ however, observed that 1 c c of a 24-hour broth culture of this organism did not produce death in rabbits when injected intraperitoneally. Guinea-pigs have been found by several investigators to be quite resistant to injections of cultures of *B. sanguinarium*. Pfeiler and Roepke state that rats, cats, and dogs are resistant to inoculations with this organism. Smith and Ten Broeck observed that 2 c c of a 24-hour broth culture of the fowl typhoid organism did not prove fatal to a chicken inoculated intravenously.

Comparatively few inoculation experiments with *B. pullorum* have been made. Rettger² and Gage consider the rabbit very susceptible to inoculations with *B. pullorum*.

TABLE 17
INOCULATION EXPERIMENTS WITH MICE

Animals	Strain	Condition and Amount of Culture	Results
2 mice	<i>B. sanguinarium</i> 2	0.5 c c of 24-hour broth culture to each	Affected slightly
1 mouse	<i>B. sanguinarium</i> 2	1 c c of 24-hour broth culture	*Found dead on next day
1 mouse	<i>B. sanguinarium</i> 47	0.5 c c of 24-hour broth culture isolated 5 days before injected	Slightly sick
1 mouse	<i>B. sanguinarium</i> 47	1 c c of 24-hour broth culture isolated 5 days before injected	*Died in 24 hours
2 mice	<i>B. pullorum</i> 9	0.5 c c of 24-hour broth culture to each	Slightly sick
2 mice	<i>B. pullorum</i> 5	0.5 c c of agar slant culture suspended in 10 c c salt solution	Slightly sick
1 mouse	<i>B. pullorum</i> 5	1 c c of agar cultures suspension	*Died after 18 hrs.
1 mouse	<i>B. pullorum</i> 7	0.5 c c of 24-hour broth culture	Slightly sick
1 mouse	<i>B. pullorum</i> 31	0.5 c c of 24-hour broth culture	Slightly sick
1 mouse	<i>B. pullorum</i> 31	1 c c of 24-hour broth culture	*Died on 4th day

* Organisms were recovered from heart blood and liver.

TABLE 18
INOCULATION EXPERIMENTS WITH GUINEA-PIGS

Animals	Strain	Condition and Amount of Culture	Results
1. Wt. 290 Gm.	<i>B. sanguinarium</i> 3	2 c c of 24-hour broth culture	*Died 20 hours later
2. Wt. 275 Gm.	<i>B. sanguinarium</i> 11	2 c c of 24-hour broth culture	*Died 18 hours later
3. Wt. 290 Gm.	<i>B. sanguinarium</i> 11	1 c c of 24-hour broth culture	Survived
4. Wt. 320 Gm.	<i>B. sanguinarium</i> 47	1 c c of 24-hour broth culture isolated 5 days before injected	Survived
5. Wt. 340 Gm.	<i>B. sanguinarium</i> 47	2 c c of 24-hour broth culture	Lost 60 Gm. in 3 days. Recovered
6. Wt. 400 Gm.	<i>B. sanguinarium</i> 11	2 c c of agar slant culture suspended in 10 c c salt	*Dead in 16 hours
7. Wt. 310 Gm.	<i>B. pullorum</i> 9	1 c c of 24-hour broth culture	Survived
8. Wt. 325 Gm.	<i>B. pullorum</i> 9	2 c c of 24-hour broth culture	Survived
9. Wt. 345 Gm.	<i>B. pullorum</i> 9	3 c c of 24-hour broth culture	*Dead in 48 hours
10. Wt. 310 Gm.	<i>B. pullorum</i> 10	1 c c of 48-hour broth culture	Survived
11. Wt. 370 Gm.	<i>B. pullorum</i> 10	2 c c of 48-hour broth culture	Survived
12. Wt. 350 Gm.	<i>B. pullorum</i> 16	2 c c of 24-hour broth culture	Survived
13. Wt. 330 Gm.	<i>B. pullorum</i> 16	3 c c of 24-hour broth culture	*Died in 4 days
14. Wt. 320 Gm.	<i>B. pullorum</i> 19	2 c c of 48-hour broth culture	*Died in 48 hours

* Organisms were recovered from heart blood and liver.

In my own work, broth cultures, and agar slant cultures suspended in 10 c c of salt solution, have been used. Inoculations were made intraperitoneally unless otherwise stated. Details of inoculation experiments are given in tables 17, 18, 19 and 20.

In addition to the experiments tabulated, white rats and pigeons were inoculated with 1-2.5 c c of 24-hour broth cultures of different strains of *B. pullorum* and *B. sanguinarium*. The rats were inoculated intraperitoneally

and the pigeons were injected intramuscularly. All the rats and pigeons survived the inoculations except that one pigeon died 4 days after being inoculated with 1.5 cc of a 24-hour broth culture of *B. sanguinarium* (47). This culture of *B. sanguinarium* had been isolated 5 days previously, from a hen which had died of fowl typhoid.

TABLE 19
INOCULATION EXPERIMENTS WITH RABBITS

Animals	Strain	Condition and Amount of Culture	Results
1. Wt. 2,580 Gm.	<i>B. sanguinarium</i> 1	1.5 cc of 24-hour broth culture	Little effect
2. Wt. 2,130 Gm.	<i>B. sanguinarium</i> 2	2 cc of 24-hour broth culture	Sick for 3 days. Recovered
3. Wt. 2,180 Gm.	<i>B. sanguinarium</i> 2	1.5 cc of 48-hour broth culture	*Died on 5th day
4. Wt. 1,275 Gm.	<i>B. sanguinarium</i> 47	1 cc of 24-hour broth culture. Isolated 5 days previously	No effect
5. Wt. 1,645 Gm.	<i>B. sanguinarium</i> 47	2.5 cc of 24-hour broth culture	*Died on 4th day
6. Wt. 1,780 Gm.	<i>B. pullorum</i> 5	2 cc of 24-hour broth culture	No effect
7. Wt. 2,250 Gm.	<i>B. pullorum</i> 7	2 cc of 24-hour broth culture	No effect
8. Wt. 2,360 Gm.	<i>B. pullorum</i> 9	2 cc of 24-hour broth culture	*Dead on 5th day
9. Wt. 1,520 Gm.	<i>B. pullorum</i> 9	1 cc of 24-hour broth culture	Survived
10. Wt. 1,075 Gm.	<i>B. pullorum</i> 22	1.5 cc of 24-hour broth culture	Little effect
11. Wt. 1,540 Gm.	<i>B. pullorum</i> 31	1.5 cc of 24-hour broth culture. Had been isolated about 2 weeks previously	Little effect
12. Wt. 2,270 Gm.	<i>B. sanguinarium</i> 56	2 cc of an agar slant culture suspended in 10 cc salt solution. Isolated about 2 weeks before	Recovered

* Organisms were recovered from heart blood and liver.

TABLE 20
INOCULATION EXPERIMENTS WITH SPARROWS

Sparrow No.	Strain	Amount and Condition of Culture	Results
1	<i>B. sanguinarium</i> 2	0.25 cc of 24-hour broth culture	Recovered
2	<i>B. sanguinarium</i> 11	0.25 cc of 24-hour broth culture	Recovered
3	<i>B. sanguinarium</i> 2	0.3 cc of 24-hour broth culture	Found dead after 4 days. Could not recover organisms
4	<i>B. sanguinarium</i> 11	0.3 cc of 24-hour broth culture	Recovered
5	<i>B. sanguinarium</i> 3	0.5 cc of 24-hour broth culture	*Died on 4th day
6	<i>B. pullorum</i> 7	0.25 cc of 24-hour broth culture	No effect
7	<i>B. pullorum</i> 9	0.3 cc of 24-hour broth culture	*Died 4 days later
8	<i>B. pullorum</i> 9	0.3 cc of 24-hour broth culture	*Died 4 days later
9	<i>B. pullorum</i> 10	0.25 cc of 24-hour broth culture	*Found dead after 4 days
10	<i>B. pullorum</i> 10	0.3 cc of 24-hour broth culture	*Died 3 days later
11	<i>B. pullorum</i> 29	1 cc of an agar slant growth suspended in 10 cc of salt solution	*Died 3 days later
12	<i>B. pullorum</i> 29	1 cc of the above suspension	*Found dead 4 days later

* The organisms were recovered from the heart blood and the liver of these animals.

Four young hens weighing between 840 and 1,350 gm. were inoculated intramuscularly with 2 cc, 4 cc, 5 cc, and 8 cc, respectively, of 5-day broth cultures of *B. sanguinarium*. Two cc of 24-hour broth cultures of *B. pullorum* (9 and 31) and *B. sanguinarium* (11 and 56) were also injected intramuscularly into hens weighing from 800 to 1,300 gm. A rooster weighing 1,875 gm. was inoculated with 2 cc of a 24-hour broth culture of *B. sanguinarium*

(57). This culture had been isolated 5 days previously from a hen that had died of fowl typhoid. All of the chickens recovered. The one that received 8 cc of the broth culture of *B. sanguinarium* (11) appeared sick for a few hours after inoculation.

From these experiments it appears that mice, rats, guinea-pigs, pigeons, sparrows, chickens and rabbits are quite resistant to laboratory cultures of these organisms. The rabbit is apparently the most susceptible. The amount necessary to kill adult rabbits varies considerably, but is generally between 2 and 3 cc of a 24-hour broth culture. It generally requires a similar amount to kill the guinea-pig. Rats and mice are quite resistant. Pigeons, sparrows and chickens were also quite resistant to the laboratory cultures of these organisms, although a pigeon was more susceptible to a freshly isolated culture of *B. sanguinarium*. The sparrow appeared to be slightly more susceptible to *B. pullorum*. In general, the pathogenesis of these two avian strains is the same for the laboratory animals.

TOXIN PRODUCTION

Smith and Ten Broeck observed that the injection into rabbits of 2 cc of the bacterial free filtrates of 5-day broth cultures of *B. sanguinarium* or of *B. pullorum* regularly produced death in a few hours. They further observed that guinea-pigs were refractory toward this toxin. The medium which they found to be the most suitable for toxin production was freshly prepared sugar-free veal broth to which was added 0.1% of dextrose. Another factor, which they considered very important, was that the depth of the layer of fluid in which the cultures were grown should not be more than 2 cm. In my work a similar medium was used except that Armour's peptone was used instead of Witte's. Results of injections of bacterial free filtrate into rabbits and chickens are given in Tables 21 and 22.

Since the results of the inoculations of toxic filtrates into guinea-pigs and white mice were largely negative, it seems unnecessary to tabulate the results. The guinea-pigs used in these tests weighed between 225 and 325 gm. Four guinea-pigs, each injected with 2 cc of the toxic filtrate of different strains of *B. sanguinarium*, survived. Two guinea-pigs, of five injected with 2 cc of the toxic filtrate of different strains of *B. pullorum*, died within 48 hours. White mice in a few cases were killed by injecting 1 cc of the toxic filtrates, but in other cases they survived such injections.

TABLE 21
INTRAPERITONEAL INJECTIONS OF 5-DAY BOUILLON FILTRATES INTO RABBITS

Strain	Amount Injected, C C	Weight in Grams of Rabbit	Results
B. sanguinarium 11	2	1,980	Breathing very difficult after 2 hours. Died an hour later. Marked rigor 5 minutes after death. Liver and spleen congested
B. sanguinarium 11	2	2,635	Breathing very difficult 2 hours later. Dead in 4 hours. Hemorrhages in gastric mucosa
B. sanguinarium 47	2	1,720	Very sick an hour later. Died in 2 hours. Marked rigor few minutes after death. Hemorrhages in gastric mucosa and under endocardium
B. sanguinarium 47	2	1,740	Very sick for several hours. Lost 160 gm. in 3 days. Recovered
B. pullorum 19	1.8	2,315	Quite sick 2 hours later. Appeared better the next morning. Recovered
B. pullorum 19	2	2,150	Found dead 20 hours later. Hemorrhages in gastric mucosa and thymus
B. pullorum 24	1.5	2,175	Died in 4 hours. Hemorrhages in thymus and gastric mucosa. Marked rigor few minutes after death
B. pullorum 24	2	2,290	Quite sick for several hours. Lost 115 gm. in 24 hours. Recovered
B. pullorum 8	1	1,655	Slightly affected. Lost 30 gm. in 24 hours. Recovered
B. pullorum 8	2	1,690	Quite sick. Lost 130 gm. in 48 hours. Normal weight in 14 days
B. pullorum 9	1.5	1,720	Died 2 hours later. Hemorrhages in thymus and endocardium. Both kidneys showed chronic interstitial nephritis
B. pullorum 9	2	1,795	Found dead 10 hours later. Hemorrhages in thymus and gastric mucosa
B. pullorum 28	2	1,875	Dead after 15 hours. Gastric mucosa and thymus hemorrhagic
B. pullorum 28	1	1,675	Quite sick. Recovered
B. pullorum 25	1.5	1,615	Quite sick. Lost 145 gm. in 24 hours
B. pullorum 25	2	1,690	Recovered
B. pullorum 32	1.5	1,880	Died in 6 hours. Hemorrhages into gastric mucosa
B. pullorum 32	1.5	1,375	Slightly affected
B. sanguinarium 3	2	1,890	Quite sick. Lost 50 gm. in 24 hours
B. sanguinarium 41	2	2,240	Death occurred in 4 hours. Hemorrhages in gastric mucosa and pericardium
B. sanguinarium 31	2	1,745	Death in 5 hours. Hemorrhages in thymus and gastric mucosa
			Death in 2 hours. Hemorrhages in the thymus

TABLE 22
INTRAPERITONEAL INJECTIONS OF 5-DAY BOUILLON FILTRATES INTO CHICKENS

Strain	Amount Injected, C C	Weight in Grams of Chicken	Results
B. sanguinarium 3	5	1,215	Slightly sick for a few hours
B. sanguinarium 11	8	1,560	Quite sick for few hours
B. pullorum 9	5	1,475	Very slightly affected
B. pullorum 31	5	1,650	Very slightly affected

In these experiments control animals have been inoculated in each case. The filtrates were tested for sterility before being injected, and blood from the heart and liver of the animals that died was tested also

for sterility. It appears that mice, guinea-pigs, and chickens are quite resistant toward the toxin. Rabbits, however, are more susceptible. The fatal dose when injected intraperitoneally is about 2 c c of a 5-day bouillon filtrate for rabbits weighing about 2,000 gm. There is some variation in the amount of toxin produced by different strains. The symptoms appear from 1-2 hours after inoculation. There may be no special symptoms or there may be respiratory and intestinal disturbances. The respiratory symptoms are manifested by marked dyspnea; the intestinal disturbances, by the discharge of many fecal balls or even liquid feces and large amounts of urine. Death may be preceded by a violent struggle, or the animal may gradually expire. Death usually occurs within 24 hours. Necropsy shows often a congested liver and spleen. Hemorrhages into the thymus have been observed in a large percentage of the cases. The heart is dilated and there may be hemorrhages under the pericardium or endocardium. Microscopic examination of the tissues shows congestion of liver, kidneys and spleen. It appears that the toxin formed by *B. pullorum* or *B. sanguinarium* produces the same effect when injected into laboratory animals. The strength of the toxin formed is approximately the same for the two organisms, but there is some variation with different strains of both organisms.

SUMMARY AND CONCLUSIONS

There is little exact knowledge at present concerning the distribution of *B. pullorum* and *B. sanguinarium*, but it appears that both cause considerable losses to poultry raisers in many parts of the United States. There has apparently been some confusion at times in differentiating *B. avisepticus* from *B. sanguinarium*. *B. avisepticus*, however, may generally be distinguished from *B. sanguinarium* by its action in milk, indol production, fermentation of carbohydrates, agglutination reaction and pathogenesis. *B. pullorum* and *B. sanguinarium* do not produce indol, generally form hydrogen sulphid in a lead acetate medium, and produce a temporary acidity in milk, but later alkali is formed. *B. sanguinarium* usually produces alkali in a shorter time than *B. pullorum*. The casein is digested by most strains of these organisms. As regards fermentation, *B. pullorum* produces acid and generally gas in several carbohydrates. *B. sanguinarium* produces acid but no gas in the same carbohydrates, and in addition produces acid in dulcitol and maltose. There is some variation in the

reactions in maltose. Some strains of *B. pullorum* produce slight amounts of acid after several days' incubation and two strains have produced acid and gas quite promptly in maltose.

Agglutination tests with these organisms have shown that there is an antigenic relation between these organisms and *B. typhosus*, *B. enteritidis*, and *B. abortus-equinus*. Such antigenic relations were not observed between these avian strains and *B. avisepticus*, *B. dysenteriae*, *B. paratyphosus* A and B, *B. suipestifer*, *B. proteus*, and *B. coli*. Absorption tests will differentiate quite readily between *B. typhosus* and these avian strains. Agglutination and absorption tests with serums of rabbits immunized toward these avian types suggest an antigenic relation between *B. typhosus*, *B. enteritidis*, and these avian strains.

Feeding experiments indicate that laboratory cultures of these organisms will rarely produce an injurious effect on laboratory animals. Two freshly isolated strains of *B. sanguinarium* and one of *B. pullorum* were also fed to various animals without producing serious effects. It has been observed from inoculations that the rabbit is the most susceptible laboratory animal to the strains studied. It generally requires from 2-3 cc of a 24-hour broth culture injected intraperitoneally to produce a fatal infection in a rabbit weighing about 2,000 gm. A few freshly isolated strains were not more virulent for rabbits, guinea-pigs, mice and rats than cultures kept in the laboratory for some time. *B. pullorum* and *B. sanguinarium* produce a toxin when grown under proper conditions which is quite poisonous to rabbits. The action of the toxin produced appears to be the same for the two strains.

These avian strains may be differentiated from *B. typhosus* by their lack of motility, their fermentation reactions in rhamnose and sorbite, and absorption tests with immune serums. Serologic tests and certain fermentative reactions may be used to differentiate these organisms from *B. dysenteriae*. They may be differentiated from other members of the paratyphoid-enteritidis group by morphologic and cultural characteristics, and serologic tests. *B. avisepticus*, as has been mentioned before, may be differentiated in many ways from *B. sanguinarium* and *B. pullorum*.

B. pullorum may be distinguished from *B. sanguinarium* by the inability of the former to ferment dulcitol, while the latter ferments

this carbohydrate. Also, the former organism does not generally produce acid in maltose and generally produces gas in several of the carbohydrates. *B. sanguinarium*, on the other hand, generally produces acid promptly in maltose and does not produce gas in any of the carbohydrates. Rhamnose is fermented promptly by *B. pullorum*, while *B. sanguinarium* does not produce acid before 48 hours' incubation. It appears, therefore, that there are sufficient differences between *B. sanguinarium* and *B. pullorum* to regard them as separate types.

THE INCIDENCE OF MENINGOCOCCUS CARRIERS IN A NAVAL TRAINING STATION DURING 1918

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With the appearance of epidemic cerebrospinal fever at various camps throughout this country shortly after mobilization in 1917, the policy of searching for meningococcus carriers practiced by British medical officers in 1915 and 1916,¹ was generally adopted. At the U. S. Naval Training Station at Great Lakes, Ill., the impetus for this work was given by a sharp outbreak of the disease in April and May, 1917. Since the carrier situation previous to this epidemic was unknown and the decline in new cases had begun before the work was far advanced, these studies threw little light on the problem at that time. As the summer advanced, however, nasopharyngeal cultures were made more and more extensively until they included routine cultures of all men entering and leaving the station and also of the probable contacts of cases, as detailed in a previous report.² In consequence, a reliable and uniform technic was gradually perfected and the general carrier index of the station determined. The subsequent occurrence of an epidemic of cerebrospinal fever that winter gave an excellent opportunity of studying its relations to meningococcus carriers and to other factors which possibly influence the variations in carrier incidence, as followed during the succeeding year.

In the preparation of the accompanying chart the curve of meningococcus carriers has been constructed on a percentage basis (of men examined) so that in spite of the variable number of men cultured each week, a constant factor is determined to indicate the relative prevalence of carriers for any particular period. The results obtained by culturing contacts of meningitis patients are included in this curve as well as those from routine cultures of incoming and outgoing men.

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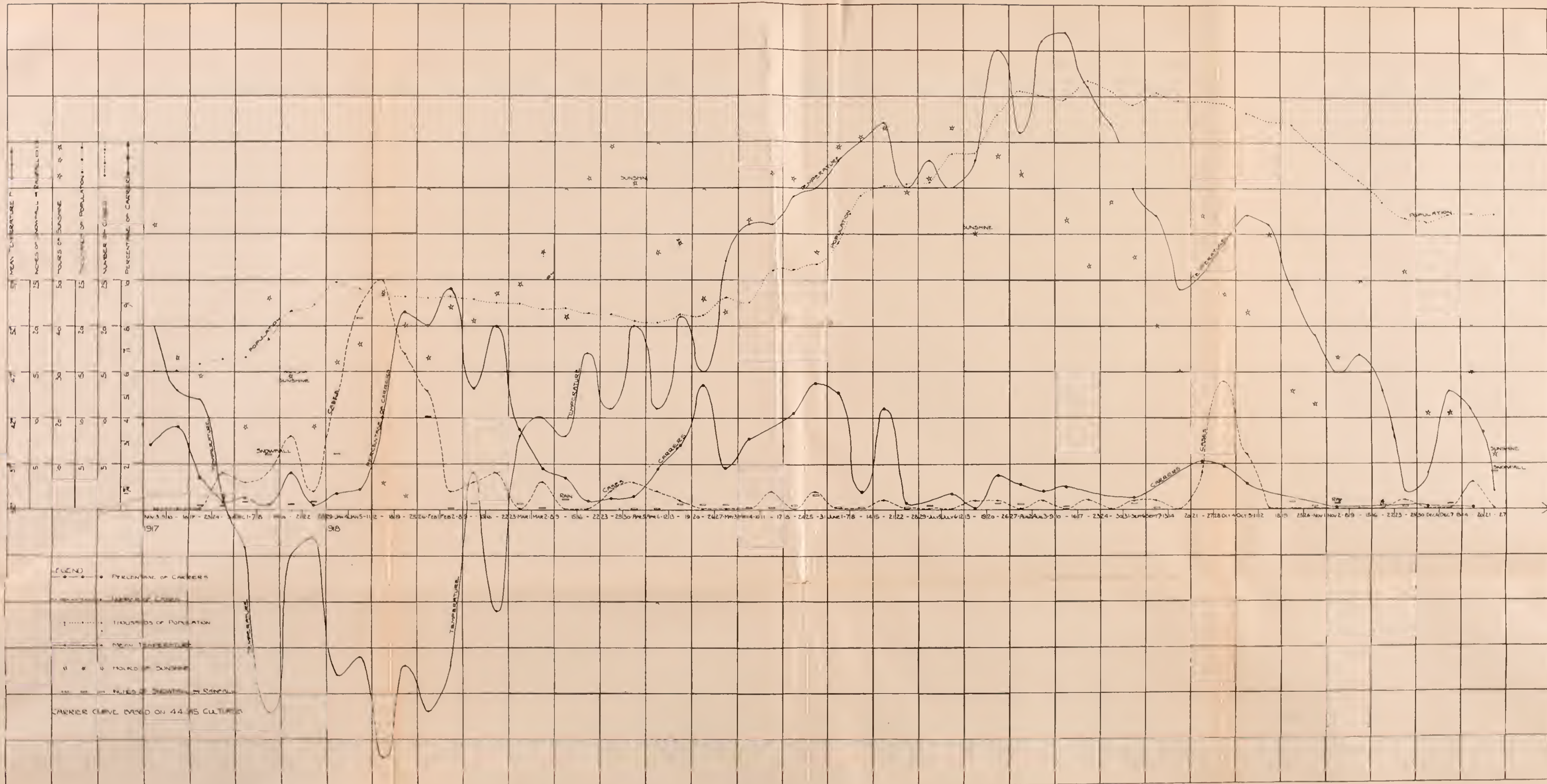
¹ Gordon: Bacteriological Measures Adopted for Dealing with the Military Outbreak in February, 1915, and Improvement Expected as the Result of Research. Special Report of the Indicated Research Committee on Bacteriological Studies in the Pathology Prevention, and Control of Cerebrospinal Fever among the Forces during 1915 and 1916. London. T. Fisher Unwin, 1917.

² Short, J. T.: U. S. Nav. Med. Bull., 1918, 12, p. 627.

A curve showing the total number of new cerebrospinal fever cases each week has been superimposed. The mean temperature each week has been shown graphically, and other weather conditions have been indicated by the total number of hours of sunshine and the total snow-fall or rainfall in inches for each week. Variations in the complement of the station are shown by a plot of the average weekly population.

It will be seen at once that although there was a sudden rise of cerebrospinal fever early in January there was no previous increase over the usual carrier index which did not begin to rise until the middle of January, two weeks later; while the peak of the carrier curve occurs three weeks after the time of the greatest case incidence, and the percentage of carriers continues high for several weeks after the case curve has begun to decline. The smaller rise in carriers and cases during December, 1917, is synchronous in this chart, but on plotting the daily figures the rise in carriers is found to come a week later. This relation cannot be demonstrated in the rise shown in October, 1918, owing to the lapse of several days during this period when no cultures were taken. On account of the time factor brought out by the comparison of these two curves, it is difficult to account for the new cases on a basis of an increase in carriers, and it seems more logical to assume that the carriers are the result rather than the cause of the epidemic. Strength is further added to this assumption by the fact that while the small number of known carriers who preceded the epidemic had been very carefully isolated, on Jan. 28, 1918 (when the carrier incidence had nearly reached its height) the restrictions on carriers were largely removed and no attempt was made to isolate those subsequently discovered. In spite of the great prevalence of unrestricted carriers when just beyond the peak of the case curve, the epidemic continued to decline. Another instance of the comparative harmlessness of carriers may be noted in this connection. A carrier (C. R. F.) who had been in isolation for several months at this time was placed on duty in an office under ordinary conditions. Cultures were taken at regular intervals and were continued in all over a year. At no time could he have been discharged under the system of obtaining three successive negative cultures, and yet, in spite of his carrying this organism for a year without any especial precautions no new cases of the disease developed among his associates, nor could any new cases be traced to this carrier.

COMPARED WITH WEATHER CONDITIONS
GREAT LAKES, ILLINOIS, 1918





The curve of weather conditions is interesting in that the period of coldest weather and heaviest snowfall, January and early February, 1918, almost exactly coincides with the period showing the greatest number of cases of the disease. It is also noteworthy that the high carrier rate after this epidemic continues until the advent of warm fair weather.

Rapid growth of the station as a factor in meningitis incidence is indicated to some extent by the population curve. It should be noted that the first rise in population which preceded the epidemic took place during cold weather at a time when added housing facilities were not readily available. The second rapid rise occurred in the summer of 1918 with no corresponding rise in either cerebrospinal fever or meningococcus carriers, but in this instance crowding was largely offset by the larger number of buildings completed at this time and the fact that tents were freely used during this warm season to house the overflow of men.

The sharp outbreak of meningitis in October is best accounted for by the lowered general resistance which followed the visitation of the influenza pandemic during September. That meningitis did not again assume the epidemic proportions that prevailed the previous winter might be due to several factors, such as the milder weather, more seasoned personnel, decreasing population or even the low carrier index which prevailed, although the reverse of the last proposition is equally tenable.

It is not the intention in this paper, however, to assign the reason for the occurrence or non-occurrence of cerebrospinal fever or meningococcus carriers, but rather to call attention to the multiplicity of factors which may be reasonably expected to operate alone or in combination to influence this incidence; to emphasize that the meningococcus carrier is only one of these factors, of which crowding, overtraining, immaturity, fatigue, exposure to weather and other factors discussed in a previous report² are probably equal, if not greater, in importance; and that to concentrate our attention on any one factor to the exclusion of a proportionate regard for the others, is to neglect some of the opportunities for controlling the situation.

A STUDY OF DREYER'S AGGLUTINATION TECHNIC IN THE ARMY

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In the diagnosis of enteric fever in civil life, the physician has three laboratory methods by which he may confirm the clinical diagnosis: (1) blood culture, (2) stool and urine culture, and (3) agglutination tests with the serum. The last test, because of its simplicity and availability, is the one most generally employed. The cultural tests have come a little more into vogue the past few years, but still are considered to be tests of necessity rather than of choice. In the Army, where the triple typhoid prophylaxis is universally administered, the agglutination reaction has been found unreliable, and laboratory methods for the most part have been limited to cultures of blood, feces and urine.

In special investigations¹ as high as 66% of positive results have been obtained in blood cultures of cases of enteric fever. It seems, however, to be the general experience that such high percentages are rarely reached.² This is especially true of army hospitals in the field, where patients may not be received until the most favorable time for securing a positive blood culture has passed. It is possible, also, that, due to the more rapid and abundant formation of antibodies, the organisms do not remain in the blood for as long a period as in uninoculated individuals.²

Stool and urine cultures have not been found very satisfactory. They are of little value in early diagnosis, and to find the organism is difficult unless it is present in relatively large numbers.

Tables 1-5 describe the experience of this laboratory with these methods. They cover the period from Oct. 2, 1918, to Feb. 11, 1919. During this period the number of patients at the Center rose from 7,000 to 17,000 and then fell to 600. The great majority of the cultures were made by Lieutenant Bigelow, who had charge of the work up to Dec. 12, 1918.

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¹ Coleman and Buxton: *Am. Jour. Med. Sc.*, 1907, 133, p. 896.

² Krumbhaar and Smith: *Jour. Infect. Dis.*, 1918, 23, p. 126.

TABLE 1
SUMMARY OF CULTURES MADE

Total number of blood cultures.....	65	
Total number of stool cultures.....	109	
Total number of urine cultures.....	27	
Total		201
Total number of patients from whom blood, feces, and urine were cultured....	12	
Total number of patients from whom blood and feces alone were cultured....	15	
Total number of patients from whom blood alone was cultured.....	25	
Total number of patients from whom feces, or feces and urine alone, were cultured	64	
Total number of patients cultured.....		116

TABLE 2
BLOOD CULTURES

		Typhoid	Para- typhoid A	Para- typhoid B	Total Positive Results
Individuals cultured once.....	43	9	1	2	12
Individuals cultured twice.....	6	0	0	0	0
Individuals cultured 3 times.....	2	0	0	0	0
Individuals cultured 4 times.....	1	0	0	0	0
Total individuals cultured.....	52				12
Total blood cultures made.....					65

TABLE 3
STOOL CULTURES

Individuals cultured once.....	78	Typhoid.....	5
Individuals cultured twice.....	10	Paratyphoid A.....	0
Individuals cultured 3 times.....	2	Paratyphoid B.....	0
Individuals cultured 4 times.....	1		
Total individuals cultured.....	91	Total positive results...	5
Total cultures made.....			109

One of the five individuals with positive stool cultures had a positive urine and a negative blood culture; one had a positive blood culture; for the remaining three, no blood or urine cultures were made.

TABLE 4
URINE CULTURES

Individuals cultured once (positives, typhoid 2).....	9
Individuals cultured twice.....	6
Individuals cultured 3 times.....	2
Total individuals cultured.....	17
Total cultures made.....	27

One of the two positive cases gave negative stool and blood cultures; one gave a positive stool and negative blood culture.

TABLE 5
CASES GIVING POSITIVE RESULTS

Blood culture alone.....	11
Both blood and stool cultures.....	1
Stool culture alone.....	3
Both stool and urine cultures.....	1
Urine culture alone.....	1
Total.....	17
Total number of individuals cultured.....	116

Krumbhaar and Smith² give the average percentage of positive blood cultures in inoculated typhoid suspects as 11%. The percentage of positives found in this laboratory is considerably higher—23.1%—and approaches the proportion given by them for uninoculated patients (22%). The percentage of positive stools found in this laboratory was low.

It must be pointed out that many ward surgeons do not appreciate the necessity for an early blood culture. In several instances, cultures were not made until the patient was convalescent, and when the report was negative the surgeons were disappointed in the laboratory for being unable to find the organism. It is worthy of note that in no case where the first blood culture was negative, did a second or third one give a positive result. Too many stools were submitted for examination as compared with blood cultures.

There were, however, a number of cases that gave definite symptoms of typhoid fever; but in which early and repeated blood cultures were negative and in which nothing could be found in the feces or the urine. The clinician was convinced that he had a case of enteric fever, but regulations for a long time prevented him from putting that diagnosis in writing without a positive report from the laboratory. It was felt by Captain Barron, the laboratory officer of this Center, that some other means of laboratory diagnosis should be invoked; under his direction I undertook the investigation here described.

The method that bears the name of the "Dreyer technic" consists, briefly, of a series of agglutinations with the patient's serum at intervals of 4-8 days, and under conditions as standard as they can be made. The results of the tests may then be plotted in a curve. A rise in the agglutination curve for one of the organisms (which is later followed by a fall) is considered as diagnostic if the curves for the other two organisms maintain the same level or fall.

Technic.—At least 2 c.c. of blood was drawn with a syringe from the median basilic vein and allowed to clot. One-half c.c. of the clear expressed serum was taken and diluted with salt solution as desired. Further graduated dilutions were made in test tubes in the usual manner.

As the Oxford "standard agglutinable strains" of killed organisms were not available, emulsions of 24-hour agar cultures of typhoid, and paratyphoid A and B were used—the same strains being used in the entire investigation. Two controls were made for each organism: one with salt solution and one with a 1:1,000 dilution of homologous stock serum. All tubes were incubated at 37 C. for 2 hours and read, using a small hand lens in reading the doubtful ones. They were then allowed to stand at room temperature over night and checked again the following morning.

A series of tests was made on 30 healthy persons that had received the triple typhoid inoculation to determine the normal titer of the serum of such individuals. For some of these, a series of two or more agglutination tests were done at intervals from 2-20 days to determine the normal variations, if any.

TABLE 6
AGGLUTINATION TITERS OF CONTROLS

No.	Date of Last Inoculation	Period Elapsed	Date at Which Serum Was Taken	Titers		
				Typhoid	Para-typhoid A	Para-typhoid B
1	April, 1918	9 mo.	1/ 9/19	+1:20	—1:20	—1:20
			1/11/19	—1:20	—1:20	—1:20
2	April, 1918	9 mo.	1/14/19	+1:20	—1:20	—1:20
			1/19/19	—1:20	—1:20	—1:20
			2/ 7/19	+1:20	+1:20	+1:20
3	July, 1918	6 mo.	1/ 9/19	+1:20	+1:40	+1:20
4	Mar. 1918	10 mo.	1/14/19	+1:20	+1:20	—1:20
			1/24/19	+1:40	+1:40	+1:20
5	Jan. 1918	12 mo.	1/17/19	+1:40	—1:20	—1:20
			1/22/19	+1:80	+1:20	+1:40
6	Aug. 1918	5 mo.	1/19/19	+1:20	—1:20	—1:20
			2/ 7/19	+1:20	+1:20	—1:20
7	April, 1918	9 mo.	1/20/19	+1:160	+1:40	+1:20
			1/26/19	+1:80	+1:40	+1:40
			2/ 7/19	+1:80	+1:40	+1:40
8	June, 1918	7 mo.	1/20/19	+1:40	+1:20	+1:20
			2/ 1/19	+1:20	—1:20	—1:20
9	Nov. 1917	14 mo.	1/20/19	+1:40	+1:40	+1:40
10	Oct. 1917	15 mo.	1/20/19	+1:40	+1:20	+1:20
11	Mar. 1917	22 mo.	1/20/19	+1:160	+1:20	—1:20
12	April, 1918	9 mo.	1/21/19	+1:320	+1:160	+1:80
			2/ 1/19	+1:320	+1:40	+1:80
13	May, 1918	8 mo.	1/21/19	+1:20	+1:20	+1:80
14	Jan. 1918	12 mo.	1/21/19	+1:180	+1:80	+1:20
15	Dec. 1917	13 mo.	1/22/19	+1:20	+1:40	+1:40
16	Febr. 1917	23 mo.	1/22/19	+1:160	+1:40	+1:20
17	May, 1918	8 mo.	1/23/19	+1:20	—1:20	—1:20
18	April, 1918	9 mo.	1/25/19	+1:40	+1:80	+1:20
19	Jan. 1918	12 mo.	1/26/19	+1:40	—1:20	—1:20
20	Mar. 1918	10 mo.	1/26/19	+1:40	+1:40	+1:40
21	Febr. 1917	23 mo.	1/26/19	—1:20	—1:20	+1:40
22	Dec. 1917	13 mo.	1/26/19	+1:20	—1:20	—1:20
23	Jan. 1918	12 mo.	1/26/19	+1:20	—1:20	—1:20
24	Aug. 1917	17 mo.	1/26/19	+1:160	+1:40	+1:80
25	Nov. 1917	14 mo.	1/26/19	+1:20	+1:20	+1:20
26	Sept. 1917	16 mo.	1/26/19	+1:80	+1:20	+1:40
27	Jan. 1918	12 mo.	2/ 1/19	+1:80	+1:20	+1:20
28	Aug. 1917	17 mo.	2/ 1/19	+1:40	+1:20	+1:20
29	July, 1917	18 mo.	2/ 1/19	+1:80	+1:20	+1:20
30	Sept. 1917	16 mo.	2/ 7/19	+1:40	+1:20	+1:20
Average.....				+1:60	+1:26	+1:23

The plus sign in the table means the greatest dilution at which agglutination occurred. Results expressed "—1:20"—mean that no agglutination occurred at this dilution—the lowest dilution used. The averages given at the foot of the table were obtained by counting all of those negatives at —1:20 dilution as zero; when two or more agglutination tests were made of the same individual the average titer was counted. Whether the higher average for typhoid over the other two organisms signifies an actual preponderance of typhoid antibodies, or whether it means that the typhoid strain used was more agglutinable than the paratyphoids, is difficult to say. Garrow³ found an average typhoid agglutination titer of 1:40 seven months after inoculation, and of 1:20 sixteen months after.

³ Jour. Path. and Bacteriol., 1909, 13, p. 331.

It will be observed that 5 of the 30 individuals tested had a titer of 1:60 or more; only one gave a typhoid agglutination of 1:320 which was found on two different occasions. This man's stool was plated out to see if he might be a carrier, but it was found negative. It will also be noticed that when two or more tests were made on the serum of the same person, it was a common experience to find small variations. In all but two cases the variation was only small, and may therefore be accounted for as due to experimental error. In Nos. 5 and 12, however, there are greater variations, and whether these were true fluctuations or experimental errors of a grosser kind, would require the experience of other investigators to decide. But in no cases were variations found as extensive as in true cases of enteric fever.

Only one vaccination experiment was attempted, as this is a phase of the subject that has been extensively worked out by others.^{2,4} The individual of this one experiment had received five triple typhoid inoculations during the months of March and April, 1918. Thus nine months had elapsed since the last inoculations. Two preliminary titrations of the serum were first made as follows:

Date	Titers		
	Typhoid Bacilli	Para- typhoid A	Para- typhoid B
1/ 9/19.....	+1:20	-1:20	-1:20
1/11/19.....	-1:20	-1:20	-1:20

On 1/11/19 he received subcutaneously 200,000,000 typhoid organisms killed with 0.5% phenol, but not heated. Further titrations were made as follows:

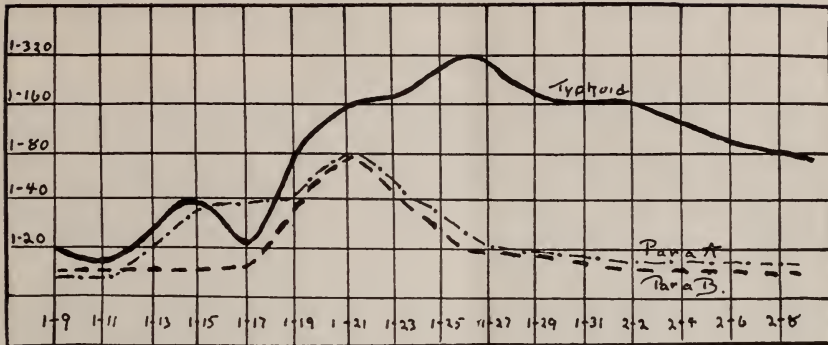
Date	Titers		
	Typhoid Bacilli	Para- typhoid A	Para- typhoid B
1/14/19.....	+1:40	-1:20	+1:40
1/17/19.....	+1:20	-1:20	+1:40
1/19/19.....	+1:80	+1:40	+1:40
1/21/19.....	+1:160	+1:80	+1:80

On 1/21/19 another injection of 200,000,000 killed typhoid organisms was given.

Date	Titers		
	Typhoid Bacilli	Para- typhoid A	Para- typhoid B
1/23/19.....	+1:160	+1:40	+1:40
1/26/19.....	+1:320	+1:20	+1:20
1/30/19.....	+1:160	+1:20	+1:20
2/ 2/19.....	+1:160	-1:20	+1:20
2/ 9/19.....	+1:80	-1:20	+1:20

⁴ Fennel: Jour. Am. Med. Assn., 1918, 70, p. 590.

Plotting these data gives the following curve:



Only two small inoculations were made. The curve, while small, corroborates the results of others, and reproduces what was actually found in clinical cases of enteric fever. While the paratyphoid curves showed an early rise, there was also a quick falling off, even while the typhoid curve was still rising. Such a curve obtained in a clinical case should be characteristic enough to permit of a positive diagnosis.

In the course of this investigation (which was not begun until Dec. 16, 1918, when patients were already being evacuated in large numbers) titrations were made of the serums of 13 patients. In the blood of 5 of these, an organism of the typhoid group had been found. These 5 will be discussed first.

1. Triple typhoid prophylaxis in June, 1917. Date of beginning of illness was not ascertained. A blood culture Nov. 25, 1918, gave paratyphoid B bacilli. Serum for agglutination obtained Dec. 20, when the patient was already out of bed and awaiting evacuation, gave titers as follows: Typhoid bacilli, — 1:50; paratyphoid A, — 1:50; paratyphoid B, + 1:50. The paratyphoid B titer was low. This case, being tested too late, is of little value.

2. Triple typhoid prophylaxis April, 1918. Admitted to hospital Nov. 15, 1918. Typhoid bacilli in blood culture Nov. 25. First agglutination Dec. 24; patient still in bed: Typhoid bacilli, + 1:400; paratyphoid A, — 1:100; paratyphoid B, — 1:100. The importance of testing lower dilutions was not realized at this time. Second agglutination Jan. 11, 1919: Typhoid bacilli, + 1:160; paratyphoid A, — 1:20; paratyphoid B, + 1:40. The first test in this case was not done until a month after the positive blood culture; an earlier test would undoubtedly have given a much higher reading; though even at this late date considerable typhoid agglutinin was still present.

3. Triple typhoid prophylaxis in April, 1917. Taken ill Dec. 23, 1918. Paratyphoid A found in a blood culture Dec. 28. Agglutination tests as follows:

Date	Titers		
	Typhoid Bacilli	Para-typhoid A	Para-typhoid B
12/28/18.....	+1:20	+1:6400	+1:20
1/ 3/19.....	+1:40	+1:3200	+1:40
1/14/19.....	+1:40	+1:1280	+1:20
1/23/19.....	+1:40	+1:1280	+1:20
2/10/19.....	+1:20	+1:640	+1:20

This case shows early rise in the paratyphoid A curve, the first agglutination being the maximum found. The patient was never very ill, and enjoyed an uneventful convalescence. It is interesting to note that altho the titer at the time of blood culture was 1:6400, it was only one-fifth that amount less than a month later, and only one-tenth after 6 weeks.

4. Triple typhoid prophylaxis August, 1917. Admitted Nov. 26, 1918. B. typhosus found in stool Dec. 2; blood culture negative. No agglutination done at this time. On the night of Jan. 22, 1919, relapse; blood culture Jan. 25 positive for typhoid bacillus. Agglutination titers: Typhoid bacilli, +1:80; paratyphoid A, +1:20; paratyphoid B, —1:20. The clinical prognosis at that time very grave; however, he rallied in a few days and is progressing favorably. Feb. 2, agglutination: typhoid bacilli, +1:640; paratyphoid A, +1:20; paratyphoid B, —1:20.

This case differs much from the preceding. In spite of the recent attack of typhoid, the agglutinating titer was low at the time of blood culture, but 8 days later it had risen from +1:80 to +1:640. The parallelism between the low initial titer and the unfavorable clinical prognosis, and then between the rising titer and the favorable prognosis, may be significant.

5. Triple typhoid prophylaxis in June, 1918; admitted Feb. 5, 1919. A blood culture Feb. 10, gave typhoid bacilli. Only one agglutination test, with this result: typhoid bacilli, +1:5120; paratyphoid A, +1:40; paratyphoid B, +1:40. This case is of the same type as case 3. At this date (Feb. 20) the patient seems to be on the road to recovery.

In addition to these five cases, agglutination tests were made in cases clinically diagnosed as typhoid, but in which blood and stool cultures were negative. Unfortunately, most of the tests were so late in the course of the disease that very little information was obtained.

Date on Which Serum Was Taken	Date of Inoculation	Date of Admission	Titers		
			Typhoid Bacilli	Para-typhoid A	Para-typhoid B
1. 12/16/18	Aug., 1918	Not ascertained	—1:100	—1:100	—1:100
2. 12/16/18	Aug., 1918	Not ascertained	+1:200	—1:100	+1:3200
3. 12/20/18	Not ascertained	Not ascertained	—1:100	—1:100	+1:3200
12/28/18			+1:80	—1:20	+1:6400
4. 12/20/18	April, 1918	Not ascertained	—1:100	—1:100	—1:100
5. 12/21/18	March, 1918	Nov. 15, 1918	—1:100	+1:400	+1:400
1/14/19			+1:80	+1:320	+1:160
6. 12/24/18	May, 1918	Nov. 20, 1918	+1:200	—1:100	—1:100
1/17/19			+1:320	—1:20	+1:20
7. 12/24/18	July, 1917	Dec. 6, 1918	+1:400	+1:400	—1:100
1/11/19			+1:320	+1:320	+1:20
8. 12/24/18	Sept., 1917	Nov. 1, 1918	+1:100	—1:100	—1:100
1/14/19			+1:160	—1:20	—1:20

As said, the important early tests were not made in these cases, which takes from the results most of their value. Cases 2 and 3, are of great interest, however, in that they show so high a titer for paratyphoid B, though both cases were in the later stages of convalescence. Cases 5 and 7 show a high titer for each of two organisms—a phenomenon that has been observed by others.² It is necessary to bear in mind that the effect of other infections, such as influenza or pneumonia, on the typhoid group agglutinins, has not been studied in this investigation. While it is not believed that "non-specific stimu-

lation" of the agglutination titer would be great enough to cause confusion, yet it would be well to study the typhoid agglutinins in such infections, to guard against a possible fallacy.

CONCLUSIONS

Another means of diagnosis of enteric fever, to supplement blood, stool and urine cultures, is highly desirable.

The agglutinating titer of the serum of healthy individuals inoculated with the triple typhoid vaccine six months or more before rarely runs higher than 1:200.

A high agglutinating titer for one of the organisms of the typhoid group permits of a positive diagnosis. Just how high this titer must be depends on the time that has elapsed since the prophylactic inoculation. A positive agglutination at 1:1000 in individuals that give a clinical picture of typhoid would seem to be of diagnostic value, if they have been inoculated for more than six months. Further tests should be made at intervals of 5 to 8 days.

In all cases in which a marked rise in the agglutinating titer for one of the organisms can be followed, while the agglutinins for the other two remain constant or diminish, a positive diagnosis may be made. A slight rise is of no diagnostic value.

The cases studied suggest strongly that information as to the prognosis may be obtained from the agglutinin curve. Thus it may be that a patient presenting a high titer will progress favorably—barring complications—while cases in which the titer is low may later develop relapse. It would be of great value to determine if a relapse could be foretold by finding low titer in cases that progress unfavorably.

Early blood culture should not be omitted. The Dreyer method should be used when the blood culture is negative, in a clinically suggestive case.

The following procedures are suggested in the order of their importance:

A. For inoculated patients:

1. Blood culture.
2. Dreyer's technic.
3. Stool and urine cultures.

B. For uninoculated patients:

1. Agglutination test and blood culture.
2. Stool and urine culture.
3. Dreyer's technic for prognosis.

A RAPID METHOD FOR PREPARING ANTIGENS FROM NORMAL HEART MUSCLE

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Since the discoveries of Landsteiner,¹ Pòrges and Meier,² and others, that alcoholic extracts of normal organs can be used as antigens, methods of preparing and standardizing antigens have been the subject of considerable study. Indeed, antigens are probably the all-important factor in the Wassermann reaction and uniformity of preparation is therefore highly desired. Thus far all attempts to standardize these preparations have not been particularly successful. This is primarily due to: (a) the variations of the lipoid content of different tissues; (b) kinds of solvents; (c) methods of extraction, and (d) periods of extractions.

The first factor is of special interest, since a comparison of plain alcoholic extracts from human hearts obtained at necropsy have given us variations in their fixative power from 1:10 to 1:180, using 0.05 and 0.1 c c of each dilution and 0.05 and 0.1 c c of the syphilitic serums. Considering the second and third factors, it is obvious that different fat solvents will yield different extractives, and these extractives will contain more or less anticomplementary and hemolytic substances. Erlandsen³ has shown that when finely divided heart muscle, dried in the air, is completely extracted with ether and then with alcohol, the first extract contains the monophosphatids while the subsequent alcohol extract contains the diamminophosphatids which were not free in the tissues but existed in the combined state. Recently Neymann and Gager⁴ advocated the extraction of the heart tissues with ether before its ultimate extraction with alcohol. According to these authors, the primary extracts contain no substances that have any antigenic value except lecithin, and this substance comprises less than one quarter of the entire weight of the ether extract, and tho it has a fairly high

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¹ Wiener Klin. Woch., 1907, 20, p. 1565.

² Berl. Klin. Woch., 1908, 45, p. 731.

³ Zeitschr. f. Physiol. Chem., 1907, 51, p. 71.

⁴ Journ. of Immunology, 1917, 2, p. 573.

binding power it is anticomplementary 1:20. The other extracts contain large amounts of fat and fatty acids, substances which are hemolytic in their properties.

Our series of extractions are comparisons between direct alcoholic and primary ether extractions followed by alcohol. As regards methods, the extractions made in the electrical shaker will certainly be stronger than those made by shaking the powder at varying intervals for the same unit of time. Finally, the period of extraction not only will influence the amount of antigenic substances, but also the amount of other extractives. We have further compared the extracts obtained in boiling alcohol, 78 C., with those obtained by an electrical shaker and those shaken at different intervals for different lengths of time at 37 C. With the exception of Landsteiner, Müller and Pötzl,¹ who extracted guinea-pig hearts with alcohol for about 10-12 hours at 60 C., the majority of investigators have applied lower temperatures during extraction.

TECHNIC

Three normal beef hearts were used. The pericardium, endocardium, larger blood vessels and fat were removed. The myocardium was finely ground and spread on glass plates and thoroughly dried at the incubator temperature. The dried material was then powdered and a total of 250 gm. obtained. The powder was divided in portions of 25 gm. and 75 cc of 95% ethyl alcohol added to each of five portions. The remaining five were extracted three times with ether using 50 cc per portion, each time for a period of 10 minutes. These portions were allowed to dry and then covered with 75 cc of alcohol. Two portions (one of ether and one of nonether extract) were extracted in a reflux condensor in the waterbath for 1 hour and two other portions for 3 hours. We have simply used a 40 inch glass tube of 3 mm. caliber as condensor. Two portions were extracted in an electrical shaker for 24 hours at room temperature; two for 2 days at 37 C., shaking 5 times a day, and the remaining two also 5 times a day for a period of 7 days. The extracts were carefully filtered and made up to 75 cc. In addition we compared these antigens with a good antigen which we have been using for many months. This antigen was treated with ether and extracted for 4 weeks with alcohol. the accompanying table gives the results of our titrations:

From these experiments it is evident that the boiled alcohol extractions are practically just as strong as those extracted under the usual conditions. The ether treated extractions have lost some binding power, especially the one boiled for 1 hour. The 24-hour shaken (ether extracted) has also lost considerable strength. All the others had more or less marked fixing power at as high a dilution as 1:200. The nonether extracted antigens contained throughout more anti-

TABLE 1
COMPARATIVE TITRATIONS OF ANTIGENS

Antigens 0.05 c c	1:10		1:50		1:100		1:150		1:200		No Serum Controls Antigens in Amounts of C C			
	Posi- tive Serum	Nega- tive Serum	Posi- tive Serum	Nega- tive Serum	Posi- tive Serum	Nega- tive Serum	Posi- tive Serum	Nega- tive Serum	Posi- tive Serum	Nega- tive Serum	0.005	0.01	0.03	0.05
1 hour boiled.....	++++	-	++++	-	+++	-	++	-	+	-	-	-	±	Inhibition
Ether extracted (boiled 1 hour).....	++++	-	+++	-	++	-	+	-	-	-	-	-	-	Inhibition
3 hours boiled.....	++++	-	++++	-	++++	-	+++	-	+	-	-	-	+	Inhibition
Ether extracted (boiled 3 hours).....	++++	-	++++	-	+++	-	++	-	±	-	-	-	-	Inhibition
24 hours shaken.....	++++	-	++++	-	+++	-	++	-	±	-	-	-	+	Inhibition
Ether extracted (shaken 24 hours).....	++++	-	++++	-	++	-	+	-	-	-	-	-	-	Inhibition
2 days at 37 C.	++++	-	++++	-	+++	-	++	-	+	-	-	-	+	Inhibition
Ether extracted (2 days at 37 C.).....	++++	-	++++	-	+++	-	++	-	±	-	-	-	-	Inhibition
7 days at 37 C.	++++	-	++++	-	++++	-	+++	-	+	-	-	-	+	Inhibition
Ether extracted (7 days at 37 C.).....	++++	-	++++	-	++++	-	+++	-	+	-	-	-	-	Inhibition
Ether extracted (4 weeks at 37 C.).....	++++	-	++++	-	++++	-	+++	-	++	-	-	-	-	Inhibition

complementary substances showing slight inhibition of hemolysis when used in 0.3 c c quantities from a 1:10 dilution, while all inhibited hemolysis when used undiluted.

All the antigens were tested in order to determine their hemolytic properties, and it was found that no hemolysis took place when using 0.3 c c of a 1:10 dilution of the antigens. When these tubes are allowed to stand at room temperature for 3 hours or more hemolysis occurs in the 0.3 c c tubes. All these extracts can be safely used in dilutions of 1:20 and amounts of 0.05 and 0.1 c c giving excellent reactions.

CONCLUSIONS

Suitable antigens can be made by extracting normal heart tissues for a period from 1-3 hours with boiling alcohol in a reflux condensor.

The value of these antigens compares favorably with those extracted by the usual methods.

A primary extraction of the dried tissues with ether results in a slight loss of their fixing power, but yields extracts containing less anticomplementary substances.

EXPERIMENTAL GAS GANGRENE

THE PROTECTION BY ANTISERUM AND ANTISERUM MIXTURES

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As Bull's antitoxin against *B. welchii*¹ was being tried out as a prophylactic and therapeutic agent in certain sectors of the British Army during the spring and summer of 1918, it seemed of interest to verify and if possible extend the experimental basis for serum treatment in gas gangrene. The claims for the potency of the antitoxin rested on experiments carried out with pure strains of *B. welchii*, and no attempt had been made to reproduce more accurately the complex bacteriologic factors found in practically all war wounds.

It is now generally known, at least in war conditions, that the clinical picture called gas gangrene, is almost never due to a pure *B. welchii* infection, but to a combined action of a variety of anaerobic organisms. It is further held by some authors that although *B. welchii* is almost universally present in the lesions of gas gangrene, it is not the most important factor in the etiology of the disease,² and that the presence of two other anaerobic bacilli, *B. edematiens* and "vibron septique," constitute a much more serious bacteriologic invasion.

The following series of experiments were planned with the idea of simulating as nearly as possible the bacteriologic conditions characteristic of war wounds, and to determine the efficacy of the different serums in combatting a mixed anaerobic infection in which the anaerobes were present in different combinations. The experiments were planned to show:

1. The relative value of *B. welchii* antitoxin and perfringens antimicrobial serum.*

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* *B. perfringens* and *B. welchii* are two names for the same organism, the former being current among the French investigators, the latter among the English and American. The *B. perfringens* antimicrobial serum is produced by the inoculation of dead and living cultures of the organism, whereas the *B. welchii* antitoxin is produced by the inoculation of sterile filtrates of the cultures.

¹ Jour. Exp. Med., 1917, 26, p. 603.

² Sacquepée, Presse méd., 1918, 26, p. 197. Weinberg et Seguin, Compt. rend. de la Soc. biol., 1915, 727.

2. The value of various antisera both singly and combined against edematous exudate produced by the inoculation of several anaerobic organisms.

3. The protective value of various antisera both singly and combined against mixed anaerobic infections.

TABLE 1

EXPERIMENT 1, INOCULATION WITH 2 M. L. D. OF B. WELCHII

Strains of Bacilli	Antiserum in CC	Results
B. perfringens....	Antiperfringens serum 4.....	Died within 24 hours
	Antiperfringens serum 4.....	Developed necrotic area in 5 days
	Antiperfringens serum 4.....	Animal lived, no local reaction
Lister B.....	Antiperfringens serum 4.....	Died within 48 hours
	Antiperfringens serum 4.....	Animal lived, no local reaction
	Antiperfringens serum 4.....	Animal lived, no local reaction
1877	Antiperfringens serum 4.....	Died within 24 hours
	Antiperfringens serum 4.....	Animal lived, no local reaction
	Antiperfringens serum 4.....	Animal lived, no local reaction
779	Antiperfringens serum 4.....	All three died within 24 hours
	Antiperfringens serum 4.....	
	Antiperfringens serum 4.....	
B. perfringens....	B. welchii antitoxin 2.....	Developed necrotic area in 48 hours
	B. welchii antitoxin 1.3.....	Developed necrotic area in 48 hours
	B. welchii antitoxin 1.....	Died in 24 hours
Lister B.....	B. welchii antitoxin 1.8.....	Died in 24 hours
	B. welchii antitoxin 1.3.....	Developed necrotic area in 6 days
	B. welchii antitoxin 1.....	Died within 48 hours
1877	B. welchii antitoxin 2.....	Died within 48 hours
	B. welchii antitoxin 1.....	Animal lived, no local reaction
	B. welchii antitoxin 1.....	Animal lived, no local reaction
779	B. welchii antitoxin 1.5.....	Died within 5 days
	B. welchii antitoxin 1.....	Died within 24 hours
	B. welchii antitoxin 1.....	Developed necrotic area in 48 hours
B. perfringens....	Normal horse serum 1.7.....	All died within 24 hours
	Normal horse serum 1.2.....	
	Normal horse serum 1.....	
Lister B.....	Normal horse serum 2.....	
	Normal horse serum 1.1.....	
	Normal horse serum 1.....	
1877	Normal horse serum 1.7.....	
	Normal horse serum 1.....	
	Normal horse serum 1.....	
779	Normal horse serum 1.9.....	
	Normal horse serum 1.3.....	
	Normal horse serum 1.....	
B. perfringens....	Culture controls	All died within 24 hours
Lister B.....		
1877		
779		

EXPER. 1

Twelve guinea-pigs were inoculated subcutaneously with *B. welchii* antitoxin, 0.5 cc per 100 gm. body weight of animal.

The same number of animals were inoculated subcutaneously with 4 cc of antiperfringens serum, irrespective of body weight.

Four days later the animals were inoculated intramuscularly with two minimum lethal doses (M. L. D.) of living, virulent cultures of *B. welchii*, a minimum lethal dose being the smallest amount of an eighteen hour culture killing a 300 gm. guinea-pig in 48 hours.

As seen from table 1, the animals were inoculated in groups of three with each of four cultures, each animal receiving the same dosage according to weight. On account of the shortage of animals only two guinea-pigs were used for each culture control, as this was considered and proved to be sufficient.

The culture control animals, and those receiving cultures and normal horse serum, died in less than 24 hours.

Of the animals receiving cultures and antiperfringens serum, five died in less than 24 hours, and one in less than 48 hours. Of the six that lived, only one gave any reaction to the culture. This animal developed an extensive necrosis of the abdominal surface.

Of the animals receiving cultures and *B. welchii* antitoxin, three died within 24 hours, two within 48 hours, and one within 5 days. Of the six remaining animals, three developed within 48 hours and one within 6 days, a liquefactive necrosis over the abdominal surface, with marked swelling of the inoculated leg. Only two of the animals failed to develop any evidence of the inoculation.

DISCUSSION

From these experiments it would seem that some protection is afforded both by *B. welchii* antitoxin and perfringens antimicrobial serum against the inoculation of pure cultures of *B. welchii*.

The fact that necrotic areas occurred at the site of inoculation in a much smaller percentage of the animals inoculated with antiperfringens serum than in those inoculated with *B. welchii* antitoxin, seems to indicate that the antibodies contained in the antimicrobial serum are more effective than the antitoxic principle contained in the antitoxin in preventing circumscribed proliferation of the organism, thus eliminating even local necrotic areas.

The protection established by antiperfringens serum, therefore, seems more clean-cut in its results than the protection by *B. welchii* antitoxin, but this apparent difference may be due to the low antitoxic content (700 units per cc) of the lot of antitoxin used.

At a later date, Major Bull personally conducted a series of experiments in this laboratory with a fresh lot of antitoxin brought over by him from America. In these experiments in contrast to the writer's results, 100% protection was afforded instead of only 50%. The following conditions differed in the two experiments: the antitoxin used

by Major Bull was dated Dec. 12, 1917, with 1,900 units per c c, the lot used by the writer Nov. 28, 1917, 700 units per c c. The difference in the dates of the two lots amounts to less than a month, but the later antitoxin contained 2.71 times as many antitoxic units per c c as the earlier lot. Notwithstanding the higher potency of the antitoxin used by Major Bull, the same number of M. L. D. (2) was given in both experiments. Furthermore, in Major Bull's experiment, although the lot of antitoxin was of higher potency than the lot used by the writer, the antitoxin was injected 48 hours before the inoculation of the cultures, instead of allowing four days to elapse as in the writer's experiment. It is impossible to know to which of these three factors: the difference in the age of the two lots, the difference in the potency of the two lots, the difference in the time between the inoculation of the antitoxins and the injection of the cultures, to ascribe the greater efficacy of the second lot of antitoxin, without more detailed work, but it would seem probable that the higher antitoxic content of the second lot might prove to be the most important factor.

From the results of Major Bull's experiment it seems apparent that an antitoxin of such potency can be produced as to give complete protection in the case of a pure *B. welchii* infection. The careful bacteriologic examination of war wounds, however, has shown that *B. welchii* almost always occurs in association with other anaerobic bacilli, and the next experiment was undertaken to determine the efficacy of the *B. welchii* antitoxin and the antiperfringens serum in the presence of combinations of anaerobic bacilli characteristic of war gas gangrene.

EXPER. 2

The production of edema in the development of gas gangrene is a very frequent occurrence. McNee and Dunn³ were the first investigators to suggest by a series of pathologic sections at various levels of an infected muscle, that the spread of the invading organisms is not preceded, as was commonly supposed, by the penetration of gas and the exertion of pressure on fresh tissues, but that the anaerobic bacteria followed in the path of a fluid or edema which was forced along the muscle fibers in advance of the infection.

This edema seemed to contain toxic principles which the body fluids were, in the majority of cases, powerless to neutralize, and

³ Br. Med. Jour., 1917, 1, p. 727.

which affected the fresh muscle tissue in such a way that the anaerobic bacteria, associated with gas gangrene could multiply freely in it.

From a prophylactic and particularly from a therapeutic point of view, it was essential that a really effective serum should be able to counteract the toxic action of the edema which always prepared the new "terrain," and without which the anaerobes could not make their way in fresh tissue.

TABLE 2

EXPER. 2, INOCULATION WITH 2 M. L. D. OF FLUID FROM GUINEA-PIGS INJECTED WITH CULTURES OF *B. WELCHII*, *VIBRION SEPTIQUE*, AND *B. SPOROGENES*

Antiserums	Antiserum in C C	Results
Antiperfringens serum	2.4 2 1.7	Died within 48 hours
<i>B. welchii</i> antitoxin.....	2.4 1.7 1.7	Died within 48 hours
Antivibron septique.....	2.4 2 1.8	Animal lived, no local reaction Died within 3 days Animal lived, no local reaction
Antiperfringens serum and anti-vibron septique, equal parts	2.5 1.7 1.7	Animal lived, no local reaction Animal lived, no local reaction Died in 55 hours
<i>B. welchii</i> antitoxin and anti-vibron septique, equal parts	2.4 2 1.8	Animal lived, no local reaction Animal lived, no local reaction Died within 24 hours
Normal horse serum.....	2.4 2 1.6	Died within 24 hours
Control animals	Died within 24 hours

The next series of experiments were, therefore, carried out with edematous fluid collected from animals inoculated with different combinations of anaerobes in the following way:

Virulent 18-hour meat cultures of the following organisms: *B. welchii*, vibron septique, and *B. sporogenes*, were mixed in equal quantities, and guinea-pigs inoculated intramuscularly with 5 c c of the mixture. On the death of the animals the edematous fluid was collected and pooled and the M. L. D. established. The edematous fluid was kept on ice, and no appreciable change in the virulence of the fluid was observed during the course of the experiment. The examination of the edematous fluid showed the presence of the three originally inoculated organisms.

* As there still exists some confusion concerning the culture known as vibron septique, it may be well to add that the culture used throughout these experiments was the non-proteolytic, highly pathogenic organism corresponding to the classification of the British Medical Research Committee.

Guinea-pigs ranging in weight from 300-400 gm. were inoculated subcutaneously as follows: 0.5 cc per 100 gm. weight.

Three with *B. welchii* antitoxin.

Three with antiperfringens serum.

Three with antivibrio septique serum.

Three with equal parts *B. welchii* antitoxin and antivibrio septique serum.

Three with equal parts antiperfringens serum and antivibrio septique serum.

Three with normal horse serum.

Four days later the animals were all inoculated into the thigh muscle with two M. L. D. of edema fluid. Three control guinea-pigs were also inoculated with the fluid alone.

The control animals and those receiving normal horse serum died within 24 hours.

The animals receiving *B. welchii* antitoxin and antiperfringens serum died within 24 and 48 hours.

Of the animals receiving *B. welchii* antitoxin and antivibrio septique serum mixture, one died within 24 hours. Of those receiving the antiperfringens serum and the antivibrio septique serum mixture, one died in 55 hours, while those animals receiving antivibrio septique serum alone, one died in 3 days (table 2).

In the same manner, animals were inoculated with *B. welchii* antitoxin, with antiperfringens serum. Four days later, the same animals were injected with two M. L. D. of edema produced by a mixture of virulent strains of *B. welchii*, *B. edematis* and *B. histolyticus*; three control animals were also inoculated at this time.

All the animals died within 48 hours (table 3). Necropsies as in the preceding experiment were made on a large proportion of the animals and in each case all the organisms present in the originally inoculated edematous fluid were isolated.

TABLE 3
EXPER. 2, INOCULATION OF 2 M. L. D. OF FLUID FROM GUINEA-PIGS INJECTED WITH
B. WELCHII, *B. EDEMATIENS*, *B. HISTOLYTICUS*

Antiserums	Antiserum in C C	Results
Antiperfringens serum	2.3 2 1.9	All died within 48 hours
<i>B. welchii</i> antitoxin.....	2.3 1.8 1.6	
3 control animals.....	...	

DISCUSSION

The experiments in tables 2 and 3 show no evidence that any protection was afforded by either antiperfringens serum or Bull's antitoxin against a mixed anaerobic infection in which *B. welchii* was associated in each case with two other anaerobes occurring commonly in war wounds.

Neither *B. sporogenes* nor *B. histolyticus* in pure culture are able to kill unless given in very large doses, whereas the pathogenic powers of both *B. edematiens* and *vibrio septique* are well known.

The death of the animals (table 2) can probably be ascribed to the presence of *vibrio septique*, and (in table 3) to *B. edematiens*. This point is brought out in table 2 by the fact that *antivibrio septique* serum alone, in spite of the presence of *B. welchii* in the combination of the infecting organisms, gives protection in 62.3%. The potency of the *vibrio septique* serum is further shown by the fact that the animals which were given equal mixtures of *vibrio septique* serum and *antiperfringens* serum, and *vibrio septique* and Bull's antitoxin — thus receiving only half of the prescribed protective dose — were also protected in 62.3% of the cases.

Unfortunately, at the time this experiment was done, no *B. edematiens* serum was available, so that the power of *B. edematiens* serum alone to protect against a mixed anaerobic infection containing *B. welchii* as well as *B. edematiens* was not tested at this point.

It seems apparent that neither *antiperfringens* serum nor Bull's antitoxin afford any protection when other pathogenic anaerobic organisms incident to war wounds are present together with *B. welchii*. These two serums must, therefore, be considered of little if any practical value in the treatment of wounds, since *B. welchii*, in almost every instance, occurs in association with other anaerobes.

On the other hand, *vibrio septique* serum shows distinct evidence that it is applicable in a practical way to war wounds, because it is able to combat alone a mixed infection in which *B. welchii* and other less virulent anaerobes are present as well as *vibrio septique*.

EXPER. 3

Twenty-four-hour cultures of the following organisms were mixed in equal parts and the 48-hour M. L. D. of the mixtures established.

Mixture 1	Mixture 2	Mixture 3
<i>B. welchii</i>	<i>B. welchii</i>	<i>B. welchii</i>
<i>Vibrio septique</i>	<i>B. edematiens</i>	<i>B. bellonensis</i>
<i>B. sporogenes</i>	<i>B. sporogenes</i>	<i>B. sporogenes</i>

It will be noticed that *B. welchii* and *B. sporogenes* are present in each of the three mixtures. This was thought advisable as these two organisms are present in a large percentage of the wounds and the desire was to make the experiment as comparable to the condition of the wound when it reaches the evacuation hospital as was possible.

Three guinea-pigs were each inoculated subcutaneously with the following antiserum, and antiserum mixtures, 0.5 c c per 100 gm. body weight.

TABLE 4
EXPER. 3, INOCULATION OF 2 M. L. D. OF CULTURE MIXTURES

Antiserums	Antiserums in C C	Culture Mixtures	Results
B welchii antitoxin.....	3.5 3 3	1	All found dead within 48 hours
Antiperfringens serum.....	2 2 2	1	All found dead within 24 or 48 hours
Antivibrio septique serum...	2 2 2	1	Animals lived, no local reaction
B. welchii antitoxin and vibrio septique serum	3.3 2.9 2.8	1	Animals lived, no local reaction
Antiperfringens and anti- vibrio septique serum	3.3 2.8 2.7	1	Animals lived, no local reaction
B. welchii antitoxin.....	3.2 3 3	2	All found dead within 48 hours
Antiperfringens serum.....	3.3 2.8 2.8	2	All found dead within 48 hours
Antiedematous serum	3.3 2.9 2.8	2	Animals lived, no local reaction
B. welchii antitoxin and anti- edematous serum	3.3 3 3	2	Animals lived, no local reaction
Antiperfringens and anti- edematous serum	3.3 2.6 2.5	2	Animals lived, no local reaction
B. welchii antitoxin.....	3.2 2.7 2.7	3	All found dead within 48 hours
Antiperfringens serum.....	3.3 2.8 2.7	3	All found dead within 48 hours
Antibellonensis serum.....	3.3 2.7 2.7	3	Animals lived, no local reaction
B. welchii antitoxin and anti- bellonensis serum	3.1 2.9 2.8	3	Animals lived, no local reaction
Antiperfringens serum and antibellonensis serum	3.1 2.9 2.8	3	Animals lived, no local reaction
3 control animals.....	...	3	Found dead within 24 hours

The animals subsequently receiving mixture 1 were inoculated as follows:

Three with *B. welchii* antitoxin.

Three with antiperfringens serum.

Three with anti-vibron serum.

Three with *B. welchii* antitoxin and antivibron septique serum, equal parts.

Three with antiperfringens serum and antivibron septique serum, equal parts.

The animals subsequently receiving mixture 2 were inoculated as follows:

Three with *B. welchii* antitoxin.

Three with antiperfringens serum.

Three with antiedematiens serum.

Three with *B. welchii* antitoxin and antiedematiens serum, equal parts.

Three with antiperfringens serum and antiedematiens serum, equal parts.

The animals subsequently receiving mixture 3 were inoculated as follows:

Three with *B. welchii* antitoxin.

Three with antiperfringens serum.

Three with antibellonensis serum.

Three with *B. welchii* antitoxin and antibellonensis serum, equal parts.

Three with antiperfringens serum and antibellonensis serum, equal parts.

Four days later, the animals were injected deep into the thigh muscles with 2 M. L. D. of the various culture mixtures. Three control animals received intramuscularly inoculations of 2 M. L. D. of cultures 1, 2 and 3, respectively (table 4). On account of the shortage of guinea-pigs, and previous experiments having shown that no protection was afforded by normal horse serum, it was not deemed necessary to make experiments with it.

DISCUSSION

In no instance was any appreciable protection established by either *B. welchii* antitoxin or the antiperfringens serum.

In all instances when antivibron septique, anti-edematiens, or antibellonensis serums were used, either alone or mixed in equal amounts with *B. welchii* antitoxin or antiperfringens serum, 100% protection was afforded in the combination containing the specific organism.

As in Exper. 2, the effectiveness is again demonstrated of anti-vibron septique serum alone to combat a mixed infection containing *B. welchii* as well as vibron septique, in this instance by the inoculation of equal amounts of broth cultures, instead of the mixed edema. In this case also the potency of vibron septique serum when diluted with equal quantities of either Bull's antitoxin or antiperfringens serum, is brought out.

In the same way, anti-edematiens and antibellonensis serums are effective against combinations containing, respectively, as the two most

pathogenic factors, *B. edematiens* and *B. welchii*, and *B. bellonensis* and *B. welchii*. These two latter serums are also potent when diluted with equal quantities of Bull's antitoxin or antiperfringens serum.

No evidence can be deduced from these experiments to show that either antiperfringens serum or Bull's antitoxin should be used singly for prophylactic purposes in the treatment of war wounds. These serums would seem of value only in a bacteriologic condition (namely a *pure* infection of *B. welchii*), which occurs so rarely as to be negligible.

The evidence, on the other hand, in favor of using vibron septique, anti-edematiens, antibellonensis serums in a practical way is striking. All three of these serums are able to protect against a mixed infection containing *B. welchii* plus the specific organism.

B. edematiens and *B. bellonensis* are considered to be the same organism by the majority of writers, so that one serum could be prepared for these two organisms. Vibron septique differs sharply from *B. edematiens* or *B. bellonensis*, and a separate serum would have to be made.

Whether the addition of either antiperfringens or Bull's antitoxin to vibron septique and *B. edematiens* (*B. bellonensis*) serums, is desirable in treating a mixed infection, or whether these two latter serums would be just as effective alone, is still to be determined. Perhaps some light can be brought on this question from the clinical data, as well as from animal experimentation.

Since it often requires a long and difficult bacteriologic technic to identify vibron septique and *B. edematiens* (*B. bellonensis*) it would seem advantageous to prepare a mixed serum from these two organisms and give it without waiting for the laboratory report.

The careful work of a few writers has shown that either vibron septique, *B. edematiens* (*B. bellonensis*) are present, although frequently overgrown, in practically every case of gas gangrene. It would seem, therefore, in preparing serums to combat these two most pathogenic anaerobic bacilli, the danger from the other anaerobic bacteria becomes a minor question.

Undoubtedly, the significance of *B. welchii* in the lesions of gas gangrene, insofar as it had been observed in civilian practice, has been exaggerated, and it was not until the importance of vibron septique and *B. edematiens* (*B. bellonensis*) was recognized in the conditions of actual warfare that a sound basis for the serum treatment of gas gangrene was established.

CONCLUSIONS

When vibron septique and *B. edematiens* (*B. bellonensis*) are present in mixed infections the prophylactic use of the specific serums even when diluted by another serum, is effective.

Neither the *B. welchii* antitoxin nor the *B. welchii* (perfringens) antimicrobial serum are of any practical value in the prophylaxis of gas gangrene caused by a mixed infection due to several anaerobic bacilli, such as is commonly found in war wounds today. No accurate investigations of the amount of antitoxin contained in the antiperfringens (*B. welchii*) serum were made, and it is therefore impossible to say whether the protection afforded in pure *B. welchii* infections by the antimicrobial serum was due to the antitoxic principle contained in it, or to other antibodies present, or to a combination of the two. Further studies will be necessary to determine whether a serum of the highest potency can be produced by the inoculation of a sterile filtrate, producing a strict antitoxin, or by the inoculation of whole cultures, producing an antimicrobial serum.

NOTE.—The culture known as Lister B was one sent by Muriel Robertson and was identical with the one used by Major Bull in his experiments, at the Lister Institute, with the *B. welchii* antitoxin from the Rockefeller Institute. The culture known as *B. perfringens* was sent by M. Weinberg of the Pasteur Institute in Paris. This culture is the "souche toxique" used by M. Weinberg in the production of his antiperfringens serum. The culture known as 1877 was isolated by the writer in this hospital from a deep buttock wound. The culture known as 779 was a stock laboratory culture source unknown. The culture of vibron septique came from M. Veillon, the culture of *B. bellonensis* from M. Sacquepee, the cultures of *B. edematiens* and *B. histolyticus* from M. Weinberg, all of the Pasteur Institute in Paris. The *B. welchii* antitoxin was obtained from the Rockefeller Institute. All the remaining serums were obtained from the Pasteur Institute.

THE FERMENTATION REACTIONS OF CERTAIN STREPTOCOCCI *

XLII. STUDIES IN BACTERIAL METABOLISM

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The group of the streptococci comprises a very important division of bacteria, characterized fundamentally by an adherence of descendants of single spherical cells in chains of a greater or lesser length. The size, and in a measure the shape, of individual units varies somewhat, and different strains or races may exhibit definite departure from the spherical morphology characteristic of the majority of members of the group.

Dynamically, the streptococci play a not unimportant part in the economy of nature; among its members are strains or races whose activities are or may be in partial opposition to those of man and the lower animals.

Some strains of streptococci appear to exist without an animal or human host. A majority live on the surface of the body or on mucous membranes of the host as "opportunists," always in communication with the exterior where escape to other hosts is readily accomplished. The normal existence of such organisms, consequently, is parasitic. The parasitic strains appear to lack the inherent power or quality of invasiveness and therefore do not ordinarily enter the tissues of the body until normal barriers which are opposed to their entrance are weakened or removed. They are "opportunists" with reference to infection. Such being the case, their association as secondary or ancillary invaders of the body in the exanthemata and other diseases is not surprising.

The type of infection induced by these "opportunists" is inflammatory in character, therefore not specific in the pathologic sense, and the portal of entry may be almost any part of the body. It is

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quite certain, furthermore, that those streptococci which do gain entrance to the underlying tissues or grow there do not succeed in escaping readily from these tissues to the surface of the body or to channels in communication with the exterior. They do not escape to other hosts in sufficient numbers, consequently, to perpetuate this infection; it is indeed doubtful whether they could penetrate the tissues of new hosts, even were escape from preceding hosts possible. In other words, parasitic strains of streptococci do not appear to be specific incitants of epidemic disease.

On the other hand, the localization of streptococci in various parts of the body in residual foci when acute symptoms have abated is an important pathologic process. It is fortunate that escape from such areas is difficult, thus preventing in a large measure the perpetuation of such strains from host to host.

Occasionally, however, strains or races of streptococci are met with which do appear to possess the power of independent invasiveness. Thus, epidemics of septic sore throat,* of streptococcus enteritis, and of streptococcus pneumonia of the epidemic type may be justly regarded as manifestations of progressive invasiveness from host to host. These instances approach most closely the fundamental requirements of a progressively pathogenic existence, namely, the multiplication of the microbe in the tissues of the host, escape from the tissues to a channel of the body in communication with the exterior, escape to the exterior, reaching a suitable portal of entry in a successive host, invasion of the tissues, multiplication within the tissues, and escape once again to a new host. In this respect, the group of the streptococci contains members of three classes: Saprophytes, which are practically without pathogenic powers; parasites, or "opportunists," and, finally, progressively pathogenic varieties.¹

A multitude of classifications of the group streptococcus are recorded. For purposes of discussion four principal avenues of approach are clearly discernible: First, a classification based on the origin of the culture, be it with reference to the host, as *Streptococcus equinus* from the horse; *Streptococcus lacticus* from the milk of the cow; from the lesion, as *Streptococcus mastitidis*, *Streptococcus scar-*

* Secondary cases do not appear to arise from contact with primary cases; transmission appears to be through milk as a vehicle.

¹ See Kendall: Bacteriology, General, Pathological and Intestinal, for a more complete discussion of saprophytism, parasitism and pathogenism.

latinae, *Streptococcus gingivae*, *Streptococcus erysipelatis*; or from a definite secretion, as *Streptococcus salivarius*, *Streptococcus ureae*.

Secondly, a classification based on changes induced in blood pigment by various members of the group. Schottmüller² appears to have been the first investigator to study the problem from this angle. As a result of work in this field, the streptococci are divisible into four principal types: Those which induce hemolysis of blood, *Streptococcus pyogenes* or *Streptococcus hemolyticus*; those which produce green colonies — *Streptococcus viridans*; those forming viscid colonies — *Streptococcus mucosus*, and, finally, cultures without obvious change in the hemoglobin and which do not form viscid colonies.

Thirdly, the use of various carbohydrates, the fermentation reactions in which furnish criteria for division into several groups. Gordon,³ and Andrews and Horder⁴ have made extensive contributions to this subject.

Fourthly, a combination of the fermentation and hemolytic reactions. The works of Holman,⁵ Lyall,⁶ and Miss Broadhurst⁷ are noteworthy in this connection. This method on the whole appears to have more nearly met the requirements of a satisfactory grouping than the others mentioned.

The work of Rosenow⁸ on the transmutation of streptococci into pneumococci for a time seriously disturbed the prevailing ideas of specificity of organisms; since this work has not as yet received confirmation, final judgment as to its possible effects on attempts to classify streptococci may be withheld. The timely statement of Smith and Brown,⁹ however, is reassuring: "Spontaneous changes in the structural characters of the streptococcus do not proceed rapidly enough, if they proceed at all, to interfere with current bacterial methods. Tendencies toward slow changes may be used as further valuable distinguishing characters."

The present investigation concerns the fermentative reactions of 356 cultures of streptococci, obtained chiefly from Camps Lee, Grant, Custer and Fort Sam Houston. These organisms were isolated from

² München. med. Wehnschr., 1903, 20, p. 849.

³ Ann. Report Local Govt. Board, Medical Officer, 1903, p. 388.

⁴ Lancet, 1906, p. 171, p. 1245.

⁵ Jour. Med. Research, 1916, 34, p. 377.

⁶ Ibid., 1914, 30, p. 487.

⁷ Jour. Infect. Dis., 1915, 17, p. 277.

⁸ Jour. Infect. Dis., 1914, 14, p. 1.

⁹ Jour. Med. Research, 1914, 31, p. 508.

cases of streptococcus pneumonia, from empyemas, blood cultures and autopsies. A smaller number of organisms were obtained from the John McCormick Institute for Infectious Diseases, the Army Medical School and Museum, and the Rockefeller Institute for Medical Research. The carbohydrates used, with the exception of C. P. lactose and maltose, were obtained through the courtesy of Dr. Hudson, Chief of the Carbohydrate Laboratory of the Department of Agriculture.

The general initial treatment of the culture was to plate it twice on rabbit blood nutrient agar (10% blood) to insure purity and to obtain its hemolytic action; then to rejuvenate repeatedly in serum broth to insure vigorous growth. The actual fermentation reactions of each strain were observed in serum broth—containing 25% of beef serum—neutral to Andrade's indicator. This is the medium Holman¹⁰ uses, and it has been found very satisfactory.¹¹ Duplicate, and frequently triplicate, determinations have been made throughout, usually with identical results. Rarely, a negative test has been followed by two positive tests, suggesting a lack of luxuriance in growth in the first instance. An apparent gain of fermenting power for different carbohydrates in successive trials has not been met with. Incubation at 37 C. was practiced for one week. Readings were made on the first, third, fifth and seventh days.

The carbohydrates used comprised the following:

- a. Pentoses: xylose, d- and l- arabinose;¹² the alcohol d. arabite.
- b. Methylpentose; rhamnose (iso-dulcitol).
- c. Hexoses: D-glucose, d-mannose, fructose, d-galactose; the alcohols, d-mannitol, d-dulcitol.
- d. Heptoses: Alpha-gluco-heptose, manno-keto-heptose, anhydro-sedo-heptose; the alcohol, perseitol.
- e. Bioses: Maltose, lactose, saccharose, trehalose (trehabiose or mycose).
- f. Trisaccharid: Raffinose (meletriose).
- g. Polysaccharid: Inulin.
- h. Glucosids: Amygdalin, salicin, arbutin.

RESULTS

A. All strains studied gave in common the following reactions:

1. Morphology—cocci in longer or shorter chains; capsules and motility not observed; young cultures gram-positive.

¹⁰ Jour. Med. Research, 1916, 34, p. 385.

¹¹ The appropriate carbohydrates are added separately as sterile aqueous solutions; one-half of 1% of each sugar has been found to be ample. The fermentation of the carbohydrate is shown both by the production of a marked red color, and by the gradual precipitation of the protein constituents of the serum (so-called acid albuminate).

¹² It should be remembered that l-arabinose, not d-arabinose, is the isomer more commonly found in nature.

2. All strains fermented d-glucose, d-mannose, fructose, d-galactose, of the hexose group; maltose, lactose, saccharose and trehalose of the biose group; salicin of the glucosid group.

3. All gave a strongly acid reaction in milk and none liquefied gelatin.

These reactions may be tentatively regarded as fundamental or group reactions for the organisms studied.

B. The following carbohydrates were not fermented by any of the strains studied:

1. Pentose group: D-arabinose, d-arabite, rhamnose.

2. Hexose group: dulcitol.

3. Heptose group: alpha-gluco-heptose, manno-keto-heptose, and perseitol, anhydro-sedo-heptose.

C. Mannitol, an alcohol with 6 carbon atoms, raffinose, a sugar containing 18 carbon atoms, and inulin are fermented by a sufficiently large proportion of strains to possess differential value. The substances themselves are important from their stereo-isomerism, and in relation to the pneumococcus group.

The glucosids, arbutin, and amygdalin, are fermented by several strains of streptococci, but the indefiniteness of knowledge of their chemical structure, together with the comparative inability of a large number of strains to utilize them, makes their importance in this connection secondary; a line of demarcation between group and individual reactions must be drawn somewhere.

D. The preliminary isolation of pure cultures from blood plates showed definitely that the action of the streptococci on the hemoglobin was a factor of considerable differential value. This is clearly indicated in Table 1.

No attempt was made to differentiate the reactivity of the cultures on hemoglobin¹³ beyond the limits mentioned. Additional studies of the chemistry of the products arising from the decomposition of hemoglobin by such strains should be made before a final discussion can be satisfactorily presented.

¹³ Attention is called to the loose use of the term "hemolytic." In the complement fixation reaction, the liberation of hemoglobin from the stroma of the red blood cell is termed "hemolytic." Possibly the term "stromolysis" would be more appropriate. On the contrary, "hemolytic" streptococci so alter blood pigment that it no longer gives a spectrum. Possibly the term "hemoclysis" would be more appropriate in this connection. An investigation of this subject is in progress at the present time. No adequate chemical explanation of the composition of green coloration induced in hemoglobin by *Strep. viridans* is available at present.

Pathogenicity and virulence for lower animals were not considered in this study, which is primarily and essentially a consideration of the ability of the organisms to utilize certain carbohydrates, together with an attempt to correlate these results with the stereo-isomerism of the substances themselves.

The accompanying table shows the results of this investigation in tabular form. For convenience and for brevity, the term "type" as used indicates the reactions possessed in common by all of the strains studied. These are:

Morphology.—Organisms typical chains of greater or lesser length, non-motile, gram-positive.

Capsules.—Not formed, lanceolate-shaped lacking.

Gelatin.—Not liquefied.

Litmus milk.—Decidedly acid, usually coagulates when heated.

Fermentation reactions.—Glucose, mannose, fructose, galactose, maltose, lactose, saccharose, trehalose, salicin, all fermented with the production of acid, but no visible gas.

TABLE 1
CULTURAL CHARACTERS OF 356 STREPTOCOCCI. (FUNDAMENTAL, OR GROUP, CHARACTERS, AS ABOVE)

Hemolytic Colonies				Green Colonies				Nonhemolytic Colonies			
Type	Man-nite	Inu-lin	Total	Type	Man-nite	Inu-lin	Total	Type	Man-nite	Inu-lin	Total
234	28	9	264	28	17	9	47	32	12	8	45
Mannite and Inulin, 7				Mannite and Inulin, 7				Mannite and Inulin, 7			
Secondary Reactions *											
Arbutin.....			16	Arbutin.....			11	Arbutin.....			7
Amygdalin.....			15	Amygdalin.....			11	Amygdalin.....			6
Raffinose.....			23	Raffinose.....			12	Raffinose.....			5
Xylose.....			4	Xylose.....			2	Xylose.....			0
L-arabinose.....			4	L-arabinose.....			2	Arabinose.....			0

* The utilization of the carbohydrates of this group by relatively few strains suggests that they are not of noteworthy importance for purposes of general classification. On the other hand, as indicators of unusual or relatively uncommon relations between stereo-isomerisms of the substrate and adaptability of the cytoplasm of the particular strain of organism, these reactions may possess unusual significance. A discussion of this particular phase is beyond the scope of the present communication.

The terms "hemolytic" colony, green colony, and nonhemolytic colony are self-explanatory.

The fermentation of mannite and of inulin, or both, is indicated, the respective numbers representing the strains which exhibit these characters in addition to the "type" characters.

SUMMARY OF FERMENTATION REACTIONS

Percentage

- 100.0 of all cultures fermented d-glucose, d-mannose, d-galactose, fructose, maltose, lactose, saccharose, salicin.
- 16.0 of all cultures fermented mannitol.
- 7.3 of all cultures fermented inulin.
- 5.9 of all cultures fermented both mannitol and inulin.
- 11.1 of all cultures fermented raffinose.
- 9.5 of all cultures fermented arbutin.
- 9.01 of all cultures fermented amygdalin.
- 1.7 of all cultures fermented d-xylose and l-arabinose.

DISCUSSION

The series of organisms studied cannot be regarded as wholly representative of the entire group of streptococci. The strains are wholly of human origin; hence, members of the bovine and equine types are lacking unless accidentally included. Furthermore, no cultures of pneumococci, of *Streptococcus mucosus*, or of *Pneumococcus mucosus* were recognized. It is improbable that any of these strains were present.

A clear majority of the organisms were isolated from streptococcus pneumonias, or from secondary processes arising therefrom; this may explain the total absence of nonlactose fermenting strains, a type emphasized by Holman.¹⁴ On the other hand, inasmuch as hemolytic, nonhemolytic and green pigment producing strains were found in this series, together with a fair proportion of mannitol and inulin fermenting types, the restrictions of origin to a rather narrow type of lesion is not reflected in the comparative variety of carbohydrates utilized.*

The pathogenicity of the organisms was not tested on animals. Several representative cultures of the hemolytic group were tested for hemolysin, using the procedure of Lyall.¹⁵ In each instance the soluble hemolysin was present in eighteen to 24-hour serum broth cultures. There appears to be no tangible relation between hemolysin production and fermentability of various carbohydrates. Inasmuch, however, as the problem of carbohydrate fermentation was the primary objective of this study, the consideration of hemolysis, virulence, and questions relating thereto, were not considered intimately.

¹⁴ Jour. Med. Research, 1916, 34, p. 377.

* Representatives of each type and group were isolated from the cultures originating in the Army Camps.

¹⁵ Jour. Med. Research, 1914, 30, p. 515.

The mechanism of the utilization of carbohydrates by bacteria is one of the most alluring in the domain of biochemistry. It brings together the fields of structural and energy requirements of single cells in their relation to the stereo-isomerism of carbohydrate, including the newly explored field of carbohydrate tautomerism, and the entire field of endo- and exo-enzymes. A solution of the mutual relations which must exist between microbes, their enzymes and their chemical nutritive environment will not only furnish a convenient method of differentiating groups of bacteria—it will almost certainly lead to a recognition of similar processes in the mutual relationships which exist between host and microbe in the broad field of immunology.

A brief consideration of the functions of carbohydrates in the nutrition of bacteria and other micro-organisms will furnish a background for a clearer discussion of the results tabulated above. In general, carbohydrates are of importance chiefly as sources of energy, although the part they play as structural elements cannot be disregarded. Advantage is taken of the energy relations in the "fermentation reactions" exhibited by bacteria. The utilization of carbohydrate for this purpose in place of protein is of fundamental importance, both culturally and dynamically.¹⁶

It is known that carbohydrates are integral parts of the nucleins both of plants and animals. Jones¹⁷ has actually analyzed the nucleic acid of yeast and identified the number and position of carbohydrates in the molecule. Bacterial cells contain nucleic acids, and there is no reason to suspect that bacterial nucleins will differ radically in this respect from nucleins derived from plants and animals. The carbohydrate radical in the protein molecule, denied by many, but indicated by the Molisch reaction, is another interesting possible example of carbohydrate (or glucose amine) forming an integral part of a structural unit. The energy requirements of bacteria greatly exceed in magnitude their structural requirements, however, and the latter phase of bacterial metabolism is ordinarily lost sight of.

Harden¹⁸ has shown quite convincingly that carbohydrate enters into a definite combination with phosphorus (forming possibly a

¹⁶ Kendall: *Jour. Med. Research*, 1911, N. S., 20, p. 140; Kendall and Farmer: *Jour. Biol. Chem.*, 1912, 12, pp. 13, 215, 219 and 465; Kendall, Day and Walker: *Jour. Am. Chem. Assn.*, 1913, 35, p. 1201.

¹⁷ *Nucleic Acids*, 1914.

¹⁸ *Fermentation*.

hexose-diphosphate: $C_6H_{10}O_4(PO_4R_2)_2$) prior to the degradation of the carbohydrate with liberation of energy for yeast cells during the process of fermentation. It is a matter of no inconsiderable interest to recognize a combination of carbohydrates with phosphorus, both for the structural nucleic acid and energy phase of metabolism in the same organism. A possible relationship between the carbohydrate-phosphorus content of yeast nucleic acid and the carbohydrate-phosphorus complex in the fermentation of sugars by yeast should be borne in mind.

In this connection, the observation of Iwanoff¹⁹ that certain molds²⁰ can actually derive their energy from thymus nucleic acid, liberating phosphoric acid and purin bases, while utilizing the carbohydrate thus set free, is of paramount interest. Additional studies, however, are required along these closely related and highly important lines. The information available at present is insufficient to correlate these observations with known bacterial activities, but the fundamental similarities in the metabolism of all living organisms makes it probable that similar or analogous phenomena will be met with in the bacteria.

Returning to known facts: It is a matter of common knowledge that bacteria can utilize for their energy requirements carbohydrates or carbohydrate derivatives which are not accessible to them under natural conditions.²¹ It is equally well known that microbes are extremely delicate appraisers of optical antipodes which are so interwoven with the chemistry of not only carbohydrates but proteins as well. Thus, many bacteria can use dextro- but not levo-isomers of the pentose and hexose series. They will pick out and ferment very small amounts of utilizable carbohydrate from a solution containing unequal amounts of two sugars. Typhoid bacilli, for example, will ferment the small amount of glucose-like carbohydrate in milk, leaving the lactose unattacked. This reaction is much more delicate than any chemical process known at present. The classic research of Pasteur²² on the assimilation of d-tartaric acid by *Penicillium glaucum* is an excellent example of the relationship existing between stereo-isomerism of an organic compound and its utilization by a micro-organism. In the experiment under consideration it was found that *Penicillium glaucum* assimilated the dextro component of racemic acid, leaving the

¹⁹ Ztschr. f. physiol. Chem., 1903, 39, p. 31.

²⁰ *Aspergillus niger* and *Penicillium glaucum*.

²¹ Harden: Nature of Enzyme Action, p. 152.

²² Compt. rend., 1858, 46, p. 615; 1860, 51, p. 298.

levo component practically untouched. More recent observations indicate that the levo component is, or may be, slowly decomposed after the dextro acid has disappeared, but the principle involved holds in either case.²³ According to Fischer and Abderhalden,²⁴ trypsin will hydrolyze alanyl-glycine ($\text{CH}_3\cdot\text{CHNH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}_2\cdot\text{COOH}$), but will not cleave glycyl-alanine ($\text{NH}_2\cdot\text{CH}_2\cdot\text{CO}\cdot\text{NH}\cdot\text{CH}\cdot\text{CH}_3\cdot\text{COOH}$).

Similarly, maltase (alpha glucase) will hydrolyze alpha-methyl d-glucosid, but not beta-methyl d-glucosid, while emulsin (beta glucose) will hydrolyze the beta but not the alpha glucosid.²⁵ The corresponding alpha and beta xylosids, containing each one less carbon atom, but having otherwise the same stereo-configuration, are unattacked by these enzymes. Observations²⁶ are recorded which would indicate that maltase does hydrolyze beta glucosids, but much more slowly than the alpha isomer.

The utilization of carbohydrates for energy by bacteria is reducible to three quite distinct phases:

(a) The preparation of the carbohydrate (if it be a glucoside, biose or polysaccharid) for assimilation. This is usually an hydrolytic cleavage, resulting finally in a hexose, as one of the final products, for example, the formation of dextrose and galactose from the hydrolysis of lactose, of dextrose from starch, of dextrose and saligenin from salicin.

(b) The assimilation of the product or products of hydrolysis.

(c) The intracellular utilization of the assimilated product, which implies, of course, that the stereo-configuration of the assimilated molecule is compatible in structure with the requirements of the microbe. Of the three phases, the last is of vital importance to the organism.

The mechanism of hydrolytic cleave of bioses, trioses and polysaccharids is not well known in so far as it relates to bacterial action. The supposition is that some bacteria produce soluble enzymes which are comparable to the soluble amylo- and saccharolytic enzymes found in the intestinal tract of man. If such be the case the interesting question arises, Is there a separate and distinct enzyme for each biose, for example, as a sucrase, a lactase and a trehalose, or does one enzyme

²³ Literature and discussion in Landoldt, Optical Rotating Power, translated by Long, p. 117.

²⁴ Ztschr. f. physiol. Chem., 1905, 46, p. 52.

²⁵ Armstrong: Simple Carbohydrates and Glucosides, p. 79.

²⁶ See Fajan's; Ztschr. f. physiol. Chem., 1910, 73, p. 25; 75, p. 232, for discussion.

fit several bioses, as a master key fits several locks? Proteolytic enzymes, as pepsin and trypsin, will hydrolyze a multitude of proteins, and it is by no means impossible to conceive of bacterial enzymes which exhibit varying degrees of hydrolytic versatility.²⁷

The fact that bacteria can break down and utilize carbohydrates to which they cannot have access in nature is suggestive. For example, trehalose is fermented by streptococci, as is salicin, yet few streptococci are ever thrown into contact with these substances.²⁸ Thus far, however, attempts to demonstrate soluble saccharolytic enzymes in streptococcus cultures have been unsuccessful.

The utilization of the products of hydrolysis of bioses and saccharids — chiefly hexoses — by bacteria and by yeasts has opened a wide field of investigation. The first extensive investigations of the relations between different sugars and their fermentability were made by Emil Fischer and his associates.²⁹ From their studies it was shown that d-glucose, d-mannose, d-fructose³⁰ and d-galactose were fermented by several varieties of yeasts, while the l-components of the same sugars were unattacked. Furthermore, d-glucose and d-mannose were readily utilizable; d-galactose, which is quite close in its configuration to d-glucose, is less readily fermented. D-talose, which bears the same stereo-isomeric relation to d-galactose that d-mannose bears to d-glucose, was not attacked by any yeast. This relationship between fermentability and configuration is most clearly shown by the yeasts, *Saccharomyces apiculatus*³¹ and *productivus*,³² which do not ferment d-galactose, although they do ferment the glucose-mannose-fructose series readily.

²⁷ It is not intended to suggest that the cleavage of protein by exo-proteolytic enzymes as pepsin and trypsin, finds its analogy in the carbohydrate-cleaving enzymes. The proteins are composed of complexes of the same set of amino acids, while carbohydrates of the biose and polysaccharid groups may be resolved into hexoses of the same empirical formula, but of considerable differences in their space arrangement. In this connection, a considerable difference may be recognized between exoproteolytic enzymes, as trypsin, which appear to be without action on living cholera vibrios in the intestinal tract, and specific immune enzymes, as cholera lysin, which will dissolve cholera vibrios in vivo and in vitro. An analogy among carbohydrates does not appear to have been demonstrated, unless the development of a specific sucrase in the blood serum of animals following subcutaneous injections of sucrose be so regarded.

²⁸ In the case of salicin, the glucose component alone is utilized, so far as is known, and cleavage would appear to be necessary for the liberation of the glucose molecule.

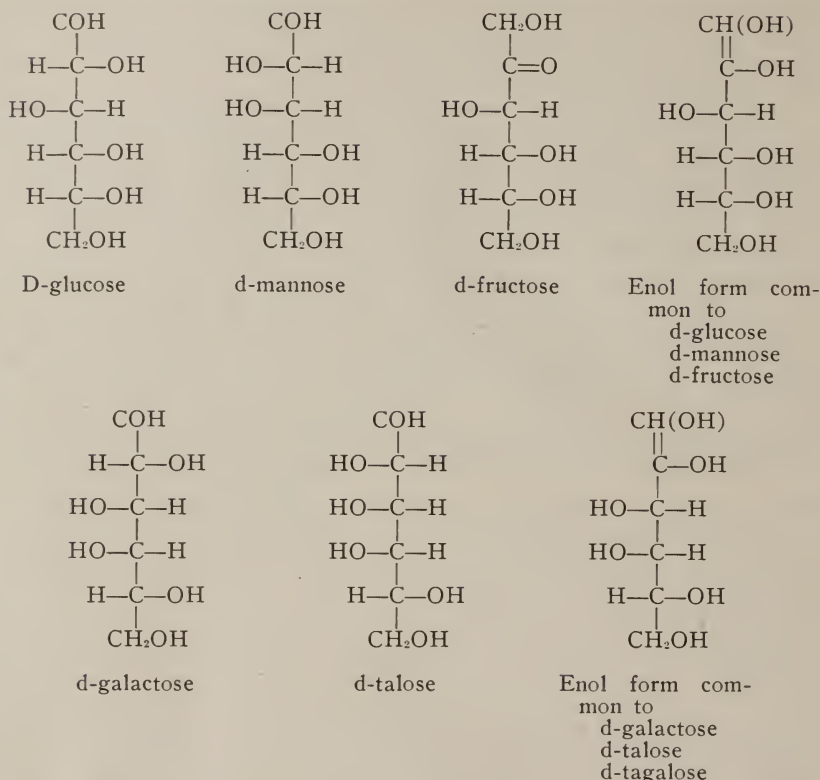
²⁹ Ber. d. deutsch. chem. Gesellsch., 1890, 23, pp. 2621, 382 and 389; 1894, 27, p. 2031; Ztschr. f. physiol. Chem., 1898, 26, pp. 60 and 89.

³⁰ Stereo-isomerism of d-group, but left rotating.

³¹ Slator: Trans. Chem. Soc., 1906, 89, p. 128; 90, p. 217.

³² Cremer: Ztschr. f. Biol., 29, p. 525.

The configuration of these sugars is as follows:



The configuration of the H and OH groupings in the carbon atoms next the primary alcohol group (CH_2OH) is the same in the d-glucose — d-mannose — d-fructose series. In d-galactose, one OH group is reversed (the Gamma C atom) and this slight change apparently suffices to reduce the suitability of the molecule somewhat for fermentability. D-talose, which bears the same structural relation to d-galactose that d-mannose does to d-glucose — that is to say, it has the two upper OH groups arranged as in d-mannose, the lower three OH groups as in d-galactose — is ordinarily not utilized at all.³³ The utilization of d-glucose, d-mannose and fructose by an organism which can ferment any one of these is presumably related to the fact that all three hexoses form the same enol. It is assumed that an

³³ Maquenne: *Les Sucres*, p. 590. It is not improbable that talose may be found to be fermentable by the more vigorous carbohydrolytic bacteria.

enzyme first converts the sugar, be it glucose, mannose or fructose, into the enol and the subsequent decomposition has its origin at the double bond of the enol. It is worthy of note in this connection that Wohl³⁴ has shown that glucose, mannose or fructose in alkaline solution gradually reaches a state of equilibrium through the formation of enol in which proportionate amounts of all three sugars are finally present in solution. If this be so, and available evidence suggests strongly that it is, then it is useless to make individual tests for fermentation of d-glucose, d-mannose and fructose, respectively. One test will settle the question.

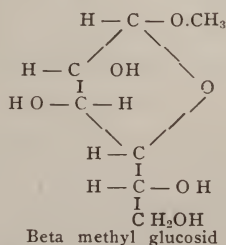
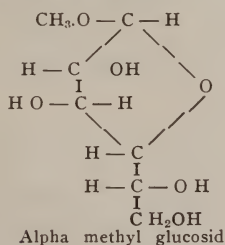
D-galactose does not form the same enol as the d-glucose series. Hence, a second test with this sugar is essential. A complication presents itself here, however. Talose and tagalose should form an enol in common with d-galactose. The few observations available suggest that talose is not fermented by any organism hitherto reported. According to the enol theory, the gradual transformation of talose to the enol form should be associated with a gradual decomposition of the enol, or possibly of d-galactose, should the enol prove refractory. It is of course possible that the products of fermentation, which are acid, may present enolization so soon that the process is overlooked. In any event, it is theoretically possible to test the entire theory of enolization and tautomerism by biological means. Experiments with the galactose — talose — tagalose series are needed to elucidate this important question.

It is significant that glucosids are not fermented by many yeasts that ferment d-glucose readily;³⁵ the terminal carbons of the glucose in the

³⁴ Ber., 1893, 26, p. 730; 1897, 30, p. 3101.

³⁵ The ordinary formula for d-glucose fails to indicate certain peculiarities of behavior of glucose solutions not accounted for satisfactorily by the simple aldehyd formula. Tollens (Kurzes Handb. d. Kohlenhydrate) has proposed a solution involving the formation of two possible aldehydols of glucose, which is now generally accepted.

The two isomers, consequently, are known as alpha and beta glucose. The methyl glucosids have the formulas:



It will be seen that enol formation is not possible in these compounds, both of which, however, are derived from d-glucose.

former case cannot undergo enolization, probably, therefore, explaining in a logical manner the inability of the organism to utilize the glucosid prior to its cleavage by another enzyme.

According to Fischer's studies,²⁴ enzymes which cleave carbohydrates exhibit similar specificity for the alpha and beta components of the glucosids, respectively; thus, emulsin splits the beta methyl dextro glucosid, but not the alpha methyl glucosid, and maltase splits the alpha methyl dextro glucosid, but not the beta methyl dextro glucosid. Neither enzyme splits the corresponding levo-glucosids. This is in harmony with the fact that levo components of fermentable hexoses are not decomposed by enzymes which cleave the dextro components.

It would appear then that the asymmetrical active agent of yeast cells attacks and ferments only those sugars which are dextro rotatory³³ and whose stereo-isomerism closely approaches that of d-glucose; similarly, the enzymes mentioned attack glucosids whose molecules approach the dextro glucosid configuration (either alpha or beta) but fail to attack the levo glucosids.

The observations above recorded deal exclusively with yeasts. In recent years much information of the catalogue type has accumulated concerning fermentation reactions by bacteria. Systematic efforts, using various stereo-isomeric series as substrates, are lacking, but one striking feature has appeared, namely, that the alcohols of the d-glucose series are less readily fermented, generally speaking, than the aldoses corresponding. Contrary to expectation, furthermore, d-mannitol, the alcohol of d-mannose, is somewhat more readily utilized than d-sorbitol, the alcohol of d-glucose. Dulcitol (inactive from internal compensatory symmetry of structure) is practically unattacked by a majority of bacteria. An explanation of this phenomenon is not available at present, but there appears to be a somewhat definite decrease in utilizability of members, otherwise the same, in the series $R.CO_2H$; $R.CHO$; $R.CH_2OH$, in the order mentioned. Thus, tartaric acid is quite generally fermented by bacteria while the corresponding alcohol, erythritol, is unattacked by ordinary bacteria.³⁷

It should be noted that this difference does not necessarily hold with bioses; for example, maltose (glucose alpha glucosid) and lactose (glucose beta galactosid) which possess potentially free aldehyd groups,

³⁶ Dextro-configuration; not necessarily dextro rotatory, however.

³⁷ Bertrand (Compt. rend. 126, p. 762) states that the sorbose bacterium, *Bact. xylinum*, transforms erythritol to erythrose.

are on the whole not much more readily fermented than saccharose and trehalose, which do not reduce Fehling's solution and have no free aldehyd group. Terminal primary alcohol radicals are certainly present in their molecules. Neither of these latter sugars is inverted by any known enzymes which will hydrolize alpha or beta glucosids.

It is a matter of common observation that the end products of the utilization of carbohydrate are almost invariably acid in character, and frequently CO_2 (action of a carboxylase) is formed as well.³⁸

Turning to the fermentation reactions exhibited by the organisms studied in this series, all of the cultures examined utilized the carbohydrates of the d-glucose series, which form a common enol, namely, d-glucose, d-mannose and fructose. So far as these observations go, the significance of this phenomenon is two-fold, namely, that a single test, using any member of the tautomeric series, will indicate what may confidently be expected from the others. Secondly, it is very probable that in general an organism which ferments one tautomerid of the dextro glucose series will utilize the other members. The mechanism of this process is not definitely known but two possibilities present themselves: Either the organism possesses an enzyme which incites enol formation prior to fermentation, or the configuration of the three members is compatible with the stereo-requirements of the cell.³⁹

Of the galactose series, only d-galactose was tried. Fermentation was distinctly slower in galactose mediums than in the glucose series, but all of the strains studied acted on this carbohydrate.⁴⁰

D-mannitol was fermented by several but by no means all the organisms. A comparison with d-sorbitol would have been interesting, because some strains of streptococci are known to utilize this alcohol.⁴¹ Inasmuch as enol formation is not possible in the alcohol series corresponding to the hexose series, a closer relation should exist between the space relations of the alcohol and the cytoplasm of the bacterium to insure fermentability than in the hexose series where a common enol furnishes a plausible reason for the mutual utilizability of the tautomerids. For this reason, the alcohols of the hexose series

³⁸ The amount of CO_2 formed is not necessarily large, and it may escape detection in ordinary procedures. It may be demonstrated readily with Van Slyke's carbon dioxid apparatus, however.

³⁹ The possibility of a reestablishment of equilibrium of three tautomerids in alkaline solution, from a single member, is not strictly a part of this phase of the discussion.

⁴⁰ It would have been very important to have d-tagalose and d-talose to complete the series of galactose tautomerids. These hexoses were not obtainable.

⁴¹ Smith and Brown: Jour. Med. Research, 1914, 31, p. 508.

appear to be of unusual interest and importance, as indicators of the versatility of the bacterial cytoplasm in relation to stereo-isomerism of the carbohydrates.

Dulcitol, the alcohol of galactose, was not fermented by any of the strains studied. In general, it is quite resistant to bacterial attack.⁴²

The bioses, maltose (glucose alpha glucosid) and lactose (glucose beta galactosid), representing, respectively, an alpha and beta compound, are readily utilizable by all the streptococci studied. Holman¹⁴ and others have found strains, chiefly from nonhuman sources, which cannot ferment the beta galactosid, however. Trehalose, a glucose derivative of glucose but without aldehyd and ketone groupings, is readily fermented, as is saccharose. Inasmuch as hydrolytic enzymes were not demonstrated in cultures of these organisms, the mere fact that the substances are utilized by the bacteria is all that can be stated definitely. Salicin, a natural glucosid, composed of glucose and ortho-oxybenzyl alcohol, was fermented by each strain studied. Arbutin, a glucose-hydroquinon compound, and amygdalin, composed of two glucose molecules with d-mandelonitrile, were utilized by a much smaller group of organisms. It is not known whether these glucosids are of the alpha or beta type; hence, nothing of a structural nature can be stated at present.

Generally speaking, those streptococci which did not produce clear areas of hemolysis around individual colonies were somewhat less versatile in the variety of carbohydrates fermented than those of the hemolytic group; those cultures which produced green colonies were on the whole the most active chemically.

The most striking feature, from the viewpoint of origin of the culture, however, is the lack of uniformity in fermentation reactions among a considerable number of strains isolated from lesions of apparently nearly identical types. This is in striking contrast, for example, with a corresponding group of typhoid bacilli where practical identity of reactions would be expected. In other words, in spite of the epidemic tendency of these streptococcus pneumonias, the organisms isolated from them failed to exhibit a corresponding uniformity in chemical reactions on carbohydrate.⁴³

⁴² Members of the coli and paracolon groups appear to utilize d-dulcitol, fermenting it with the production of gas and acid.

⁴³ It is suggestive that typhoid bacilli incite a definite clinical syndrome with a well marked pathology. Streptococci, as a rule, do not incite such definite unified syndromata and usually give rise to inflammatory reactions not restricted to definite organs or tissues.

Inasmuch as the products formed from the intracellular utilization of any and all of the carbohydrates are qualitatively similar, however, this means comparatively little from the standpoint of pathogenesis. The products arising from the action of the organisms on protein, on the contrary, are almost certainly of paramount importance in relation to the clinical disease and pathologic lesions, and the specificity of pathogenesis of the streptococci, therefore, is rather to be sought for in the domain of protein than carbohydrate chemistry.

SUMMARY

Carbohydrates are important in bacterial nutrition chiefly as sources of energy. They spare or protect nitrogenous constituents in so far as energy requirements are concerned, but they do not and cannot substitute for nitrogenous substances, which are indispensable for the structural requirements of the organisms. It is probable that certain members of the group also become integral parts of structural units, as for example, components of nucleins.

Carbohydrates are biochemically of extreme interest in bacteriology through the relations which exist between the stereo-isomerism of members of the group having the same empiric formula, and their utilizability by various types of bacteria.

Carbohydrates possess value in the classification of bacteria through the fact that a definite relationship apparently exists between the stereo-configuration of various definite groups of these substances and the ability of the organisms to ferment them. In other words, the "fermentation reactions" of many kinds of bacteria are a means of their identification.

The significance of a classification of bacteria based on fermentation reactions, therefore, depends on a recognition of the relation between the space arrangement of the carbohydrate and a fundamental capacity of the cytoplasm of the microbe to dissociate it with the liberation of energy for the cell.

The unerring specificity of these reactions possess theoretical interest in that a careful selection of bacteria as reagents of extraordinary delicacy opens a way for the testing of important theories relating to carbohydrates, as, for example, the formation of enols, and of tautomerism.

The products of fermentation produced by a great majority of bacteria are qualitatively very similar, irrespective of the carbohydrate

fermented and of the organism inciting the process. Thus, diphtheria, typhoid, colon bacilli, streptococci and staphylococci produce varying amounts of organic acid, chiefly lactic, from a great variety of sugars.⁴⁴ The specificity of action of these bacteria, therefore, would appear to depend principally on the products arising from the utilization of protein, not only for structure but for energy as well.⁴⁵

The classification of bacteria, including the streptococci, therefore, on the basis of fermentation reactions makes it possible to separate them into convenient and distinct groups which have divisional value.

There is no clearly discernible relationship, however, between cultural grouping based on carbohydrolysis and pathogenesis.

⁴⁴ In other words, they form the essential products of "sour milk" in utilizable carbohydrate-protein media.

⁴⁵ Kendall: *Am. Jour. Med. Sc.*, 1918, 156, p. 157.

IS HEMOGLOBIN ANTIGENIC?*

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A review of the literature on the properties of hemoglobin as a possible antigen shows that the results obtained by various workers differ.

Thus in work reported by Leblanc,¹ a precipitin for beef hemoglobin was obtained by immunizing a dog with a preparation obtained by saturating laked, washed, red cells with ammonium sulphate after removal of globulins and stromata. This preparation was by no means pure since no attempt was made to free the hemoglobin from possible contamination by other proteins which may have been carried down by the crystals. Levene² was unable to produce either a lysin or an agglutinin for red cells by immunization with twice crystallized hemoglobin. Ford and Halsey,³ working with various fractions of the red cell, were unable to demonstrate antibodies—precipitins, lytic or agglutinating substances in rabbits or anaphylaxis in guinea-pigs injected with dog or hen hemoglobin recrystallized 4–5 times from 25% alcohol. These authors used special precautions to exclude protein contamination and their results appear to be more reliable than other workers in this field. Schittenhelm and Weichardt⁴ and Dittrich^{5a} carried out experiments with both hemoglobin and the protein fraction of that molecule, globin. The former report two anaphylaxis experiments on guinea-pigs, using horse hemoglobin prepared by precipitation with ammonium sulphate according to the method of Schulz⁵ and recrystallized three times. One sensitized animal died on receiving an injection of 100 mg. 14 days after the initial injection while the second animal showed merely a rise of 2 C. in body temperature, which however was but little more than the normal variability. Hemoglobin (100 mg. injected intravenously into guinea-pigs) was nontoxic; no influence on the blood pressure of rabbits was noted. Contrasted with these results are their experiments carried out with globin. This substance, like protanin and thymus histone, was found to be toxic.⁶

Extensive anaphylaxis experiments with dog and beef hemoglobin have been reported by Bradley and Sansum.⁷ They found that not only is hemoglobin

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¹ La Cellule, 1901, 18, p. 337.

² Jour. Med. Research, 1904, 12, p. 191.

³ Jour. Med. Research, 1904, 11, p. 403.

⁴ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1912, 14, p. 609.

^{5a} Arch. f. exper. Path. u. Pharm., 1892, 29, p. 247.

⁵ Ztschr. f. physiol. Chem., 1898, 24, p. 449.

⁶ Schittenhelm, A., and Weichardt, W.: München. med. Wehnschr., 1912, 59, p. 1089. Cf. also, Thompson, W. H.: Ztschr. f. physiol. Chem., 1900, 29, p. 1; Gay, F. P., and Robertson, T. B.: Jour. Exper. Med., 1912, 16, p. 479; Taylor, A. E.: Jour. Biol. Chem., 1908, 5, p. 311; McCrudden, F. H.: Amer. Chem. Soc. Abstr., 1912, 6, p. 1181.

⁷ Jour. Biol. Chem., 1914, 18, p. 497.

antigenic with regard to its ability to produce anaphylaxis in sensitized guinea-pigs, but it is also specific for the species from which it was obtained. The hemoglobin was prepared from laked, washed, red cells to which toluol had been added and the crystals washed with a little water and dried. The preparation used for the second injection was less pure. A possible criticism of these experiments is that if the hemoglobin used by Bradley and Sansum for sensitization was slightly contaminated with foreign protein and since the preparation used for the second injection was less pure, the anaphylaxis obtained by them may have been due, not to the hemoglobin, but to the contaminating protein. Rosenau and Anderson⁸ found that a millionth of a c c of horse serum was sufficient to sensitize guinea-pigs and Wells⁹ states that one twenty millionth of a gm. of pure egg albumin will sensitize sufficiently to produce typical anaphylactic symptoms, while one millionth of a gm. will sensitize fatally. Bradley and Sansum while admitting that globin is toxic and nonspecific† (since it is nonantigenic) and that the relatively simple hematin portion of the hemoglobin molecule may be the same in all hemoglobins, believe that the specific differences found by them in various hemoglobins must be found in the protein or globin fraction which, however, are lost in the ordinary acid cleavage. To accept this idea we would have to assume that the union of nonantigenic globin with the nonprotein substance hematin, as combined in hemoglobin, produces an antigenic compound. Doubt is cast on this work by the fact that the animals sensitized to the purest dog hemoglobin used reacted also to dog serum.

Thomsen¹⁰ prepared horse hemoglobin by crystallizing twice from 25% alcohol and was able to demonstrate specific anaphylaxis in guinea-pigs sensitized with this hemoglobin. He believes that anaphylaxis induced by red cells is largely due to the hemoglobin. Our own observations¹¹ in this field lead us to believe that a protein other than hemoglobin is the antigen concerned in the production of specific immune bodies for the red cell.

In considering the properties of hemoglobin as a possible antigen two questions arise, namely: (a) does combination of globin which is nonantigenic, with hematin, which is not a protein and is also nonantigenic,¹² as found in hemoglobin, result in the latter substance acting as an antigen? (b) Is hemoglobin, owing to the toxic constituent globin, likewise toxic? That globin is nonantigenic and toxic appears to be well established, although experiments have been reported by Browning and Wilson¹³ to the effect that globin is not only antigenic, but shows marked species specificity. The later experiments of Gay and Robertson¹⁴ and Schmidt¹⁵ contradict this and confirm the fact

⁸ Bull. Hyg. Lab., U. S. P. H. S., 1906, No. 29.

⁹ Jour. Infect. Dis., 1908, 5, p. 449.

† This may not be strictly true. Thus Kossel, *Ztschr. f. physiol. Chem.*, 1913, 88, p. 163, states that the content of amino acids varies in different protamins, and is specific for the species from which the preparations are derived.

¹⁰ *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, 1909, 3, p. 539.

¹¹ Bennett, C. B., and Schmidt, C. L. A.: *Jour. Immunol.*, Vol. 4. To appear shortly.

¹² Gay, F. P.: Personal communication.

¹³ *Jour. Path. and Bacteriol.*, 1909, 14, p. 174.

¹⁴ *Jour. Exper. Med.*, 1913, 17, p. 535.

¹⁵ *Univ. of Cal. Pub. Path.*, 1916, 2, p. 157.

that globin is nonantigenic and likewise toxic. Combination of globin with hematin appears to markedly, if not entirely, decrease the toxic action of this protein as a review of the early literature on hemoglobin by Kuntzen and Krummacher¹⁶ shows. These workers injected horse hemoglobin crystallized three times from 25% alcohol in doses of 0.5-1.3 gm. per kg. into guinea-pigs and rabbits without toxic effects. Barratt and Yorke¹⁷ likewise confirm these results in a general way, although they used only laked red cells free from stromata.

Our experiments were initiated in the belief that the positive results obtained by other workers can be ascribed to impurities in the preparations of hemoglobin and for this reason our preparations were crystallized repeatedly in order to include as little protein contamination as possible. Crystalline proteins exhibit marked ability to carry down the menstruum from which they are deposited, the observations of Schulz and Zsigmondy¹⁸ showing that egg albumin must be recrystallized 3-6 times to insure a preparation of reasonable purity. We have made use of the well-known fact that reduced hemoglobin is more soluble than the oxyhemoglobin for the purpose of recrystallizing hemoglobin. For our experiments three preparations were employed, each crystallized by a somewhat different method. These we shall designate as A, B and C.

For preparation A we proceeded as follows: Oxalated dog blood was freed from serum and washed six times with equal volumes of physiologic salt solution, the red cells being thrown down by centrifuging and the supernatant fluid removed each time. The corpuscles were then laked with 3 volumes of water and the insoluble stromata removed by centrifuging. To this solution sufficient alcohol was added to make a concentration of 25% and the mixture cooled by the use of ice and salt, causing crystallization of the hemoglobin. The crystals were thrown down by centrifuging, the supernatant fluid decanted, and to the crystals 3-4 volumes of distilled water added. The oxyhemoglobin crystals were brought into solution by passing in CO₂, the reduced form being much more soluble. This solution was centrifuged to remove insoluble material, oxygen passed through it for an hour, and placed in a freezing mixture as previously, again causing crystals of oxyhemoglobin to deposit, which in turn were separated from the mother liquor by centrifuging. This method of crystallization was repeated 5 times. Before the 5th crystallization the hemoglobin solution was passed through a porcelain filter to remove any possible suspended material. A portion of these crystals were air dried. These did not completely redissolve, due probably to some denaturization. Another portion which was subsequently used as antigen in the fixation tests was kept frozen until used.

¹⁶ Ztschr. f. Biol., 1900, 40, p. 228.

¹⁷ Ztschr. f. Immunitätsforsch. u. exper. Therap., 1912, 12, p. 333; Brit. Med. Jour., 1914, 1, p. 235. Cf. also Sellards and Minot, Jour. Med. Research, 1917, 37, p. 161.

¹⁸ Beitr. z. chem. Phys. u. Path., 1902, 3, p. 137; cf. also, Robertson, T. B.: The Physical Chemistry of the Proteins, New York, 1918, p. 316.

For immunizing rabbits the mother liquor from the last crystallization was used. To this, phenol to make a concentration of 0.25% was added as a preservative and the solution kept in the ice-chest. In another preparation the initial addition of alcohol was omitted and the hemoglobin crystallized from the aqueous solution. The yield of oxyhemoglobin crystals by this method is small, due to its solubility even at low temperatures and unavoidable losses in centrifuging, which could not be done at low temperatures. In the next preparation use was made of alcohol to decrease the solubility of the oxyhemoglobin which gave a relatively better yield.

For the preparation of specimen B we proceeded as follows: To the hemoglobin solution obtained as in the preceding preparation by hemolysis of washed red cells (dog) and removal of the insoluble stromata, sufficient alcohol was added to make a concentration of about 15%. The solution was then saturated with oxygen and placed in a freezing mixture and the crystals allowed to separate. They were then centrifuged off, 2 volumes of water added and CO₂ passed in to obtain a solution. Any insoluble substance remaining was centrifuged off. To the solution sufficient alcohol was added to make a concentration of 15%, the solution saturated with oxygen and crystallized as before. The hemoglobin was crystallized 6 times in the above manner except that in the last crystallization no alcohol was used. Addition of alcohol to the solution increases the yield of hemoglobin crystals. It is likewise necessary to completely saturate the solution by passage of oxygen for several hours to obtain maximum yields. Part of the hemoglobin was dissolved, phenol added to make a concentration of 0.25%, kept on ice and used for the injections. The remainder was kept frozen until used as antigen for the fixation experiments. The two preparations described above at the end of a month's time still gave the usual tests for hemoglobin.

Our third preparation C was made essentially in accordance with the directions given by Schulz.⁵ To the solution of red corpuscles freed from stromata and prepared as in the previous methods, and kept cold, sufficient ammonium sulphate was added to bring the solution to half saturation and the globulins filtered off. On raising the temperature to 40 C. and usually by the addition of a little more ammonium sulphate, hemoglobin crystals formed and could be filtered off. These crystals differed markedly from those obtained by the previous methods and instead of being red, were brown, irrespective of whether oxygen or carbon dioxid was passed into the solution. In all probability this preparation was methemoglobin as stated by Schittenhelm and Weichardt.⁴ The crystals were dissolved in water and recrystallized, this being repeated three times. Removal of the ammonium sulphate by successive addition of weak alcohol and finally washing with absolute alcohol and ether, caused the hemoglobin to become insoluble; even on addition of traces of alkali, it was difficult to obtain a satisfactory solution. To overcome this another portion of the crystals was allowed to air dry. As noted by Schulz, in this procedure the ammonium sulphate largely separates from the dark brown mass and the surface appears gray and fluffy, somewhat in appearance to hoar frost. By successively removing this top layer the whole mass of hemoglobin was brought into this condition. By this procedure a large part of the ammonium sulphate separated from the hemoglobin crystals and by rapidly draining with successive additions of ice water, could be largely removed. Before air drying this procedure would have been impossible due to the rapid solution of the hemoglobin. The preparation was then air dried. It, however, still contained a small amount of ammonium sulphate. For the antigen in the fixation experiments two preparations of hemoglobin were used: one prepared by the above

method but crystallized 6 times, and the other preparation B crystallized 5 times. Since the Schulz method of preparation in our hands appeared less satisfactory than our other methods, only two rabbits were injected with this preparation.

For the immunization experiments healthy rabbits, not previously used, and from different sources, were usually given 3 successive injections at 1- or 2-day intervals and after about 5 days this was repeated. Ten days after the last injection the rabbits were bled and fixation experiments carried out with the inactivated sera. With various pure proteins we have never failed to obtain immune bodies when animals were immunized according to the above method. Using antigen (A) three rabbits were given 6 injections, the total quantity of hemoglobin injected being: Rabbit N, 0.780 gm.; Rabbit S, 0.650 gm.; and Rabbit D, 0.680 gm. Alexin fixation experiments with the inactivated sera were carried out using the following doses: 5% sheep cells, 0.2 cc; rabbit vs. sheep cell amboceptor, 0.2 cc; 10% alexin, 0.2 cc (1¼ units); antigen (crystallized 5 times and kept frozen), 0.15 cc (one quarter of the minimum inhibiting dose); sera from rabbits injected with hemoglobin in amounts of 0.4, 0.3, 0.2, 0.1 cc of 1:10, 1:50, etc. dilutions, and the volume brought to 1.2 cc with salt solution. Readings were taken after the usual half-hour incubation and after standing in the ice-chest overnight. Positive fixation with these sera was not obtained. Sera N and S in a dosage of 0.4 cc of 1:10 dilution caused a slight inhibition of hemolysis but insufficient to be called positive. In dilutions and doses lower than 0.4 cc of 1:10, slight inhibition of hemolysis was caused by the sera alone. Precipitin tests were likewise negative. The sera of these rabbits did not contain an agglutinin nor did they, in presence of alexin, cause lysis of a suspension of dog cells. It is apparent that repeated injection of hemoglobin preparation A did not produce immune bodies in the sera of these rabbits. Two normal guinea-pigs were sensitized with 10 and 20 mg. of hemoglobin injected subcutaneously and a month later given a second injection of 150 mg. intraperitoneally. In the observations for symptoms of anaphylaxis we have followed the scheme used by Wells and Osborne¹⁰ in their protein studies. No general symptoms of anaphylaxis were shown by the animals. Pig (a) may be said to have shown a possible slight reaction (fall of several degrees in body temperature) as a result of the second injection while the change in the second animal was within the normal variability of temperature change when proteins are injected.

Using hemoglobin preparation B, three rabbits were injected as follows: two received 7 injections of 200 mg. each and the third 5 injections of the same dosage. Fixation experiments were carried out as before using two antigens, (a) the solution which was used for the injections, (b) the preparation which had been kept frozen. Using dosages and dilutions of 0.4 cc of 1:10 and upward of inactivated sera, no inhibition of hemolysis was observed. Precipitin tests were likewise negative. These sera contained no sensitizer which in the presence of alexin could cause hemolysis of dog cells. The serum of rabbit C in a dilution of 1:10 caused agglutination of dog cells, but this was probably due to the occurrence of a normal agglutinin rather than production through immunization.

Three guinea-pigs were sensitized with 1 mg. each of this hemoglobin preparation given subcutaneously. One month later each animal received 100 mg. injected intraperitoneally. The animals showed no symptoms of anaphylaxis. In two animals the fluctuation in body temperature after injection was within

¹⁰ Jour. Infect. Dis., 1911, 8, p. 66.

the normal variability, in the third it was slightly greater. Marked reactions such as have been reported by Bradley and Sansum and which one ought to expect with a soluble substance such as hemoglobin, were not obtained.

Using hemoglobin prepared according to the Schulz method (preparation C) two rabbits were given 7 injections each, a total of 950 mg. The inactivated sera from these animals were used to carry out fixation experiments using as antigens, preparation C crystallized 6 times and B crystallized 5 times. No fixation was obtained. The precipitin tests were also negative.

As has been previously mentioned, it is regarded by most observers that hemoglobin is nontoxic in moderate doses. We have confirmed this fact. Two guinea-pigs were injected with 70 mg. each of hemoglobin B intracardially. No symptoms were exhibited by the animals. Two other pigs were injected with 200 mg. of the same preparation intraperitoneally. No symptoms and very little effect on temperature were noted. When it is recalled that Gay and Robertson¹⁴ obtained severe reactions in animals injected with 10 mg. of globin intravenously, it is apparent that the combination of globin with hematin as found in hemoglobin has the effect of rendering the protein non-toxic.

SUMMARY

Hemoglobin was prepared by three methods, one by crystallization from ammonium sulphate as described by Schulz, and the other two by precipitation as oxyhemoglobin (with and without addition of alcohol) at low temperatures and resolution by reduction. Each of the three preparations was recrystallized a number of times.

Rabbits were immunized with each of the above preparations and both fixation and precipitin tests carried out. These were negative. In the sera of these rabbits no sensitizer was found, which in the presence of alexin, cause a hemolysis of dog cells. Agglutinins were likewise not produced. Hemoglobin failed to sensitize guinea-pigs for the anaphylaxis reaction. The conclusion that hemoglobin is nonantigenic seems warranted.

Doses of hemoglobin very much larger than the amount of globin which will cause toxic symptoms were injected both intravenously and intraperitoneally into guinea-pigs and toxic symptoms were not shown. Combination of toxic globin with hematin as occurs in hemoglobin renders it nontoxic, but does not, as in the instance when globin is combined with casein,¹⁴ give, when rabbits are immunized, antibodies for itself.

MOUSE PATHOGENIC PNEUMOCOCCI AND STREPTOCOCCI IN THE SPUTUM IN PULMONARY TUBERCULOSIS

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The classification of pneumococci on the basis of immunological reactions has led to the discovery of a number of important facts, and has changed some of our conceptions regarding the pathogenicity of this organism especially for man. The progress in classifying the streptococci also is of great interest, especially with respect to the pathogenicity of this organism. Recently, Avery and Cullen¹ have been able to divide the hemolytic streptococci into two distinct groups, from human and bovine sources, by the final hydrogen ion concentration attained in culture. Corper, Donald and Antz² found that in the majority of cases of pulmonary tuberculosis pneumococci and streptococci were present in the sputum, the most common pathogenic varieties being pneumococci (types II and III) and streptococci of the viridans group. A few type I pneumococci and hemolytic streptococci were found. Approximately 30% of the sputums revealed so-called pathogenic varieties of pneumococci (types I, II and III), and hemolytic and green-producing streptococci. It is noteworthy that of the pneumococci type IV was more commonly found than any other, and of the streptococci the saprophytic more commonly than others. About 37% of the 216 sputums contained no pneumococci or streptococci. Uniformly negative results were obtained from the examination of the blood during life of 216 cases of pulmonary tuberculosis. These results, with those from observations during the influenza epidemic, also on cases of pulmonary tuberculosis, led to the conclusions that organisms, especially pneumococci and streptococci, found in the sputum in pulmonary tuberculosis, ordinarily play an insignificant part in the pathogenesis of this disease. It is interesting here to note also the results of Pritchett and Stillman³ in lobar

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¹ Jour. Exper. Med., 1919, 29, p. 215.

² Jour. Infect. Dis., 1919, 24, p. 496.

³ Jour. Exper. Med., 1919, 29, p. 259.

pneumonia and influenzal bronchopneumonia. They found that, whereas usually pneumococci of types I and II are associated with over 60% of the cases of lobar pneumonia, in the bronchopneumonia complicating influenza these types are rare in comparison with types III and IV, which are found normally and commonly in the mouths of healthy persons and only occasionally in lobar pneumonia. Stillman⁴ further has found that the atypical type II pneumococci also fall into a parasitic group, associated with lobar pneumonia and a saprophytic group, found in normal mouths.

Since the pneumococcus and streptococcus are both highly pathogenic to the mouse it was deemed advisable to study the sputum of cases of pulmonary tuberculosis by injecting it into mice (intraperitoneally) and culturing and typing directly from the peritoneal fluid, and then to compare these results with those previously obtained by cultural methods. For comparison, the saliva of cases of pulmonary tuberculosis and the saliva and sputum of individuals free from abnormal clinical pulmonary conditions were studied also.

The sputum was obtained after rinsing the mouth with sterile salt solution several times and was delivered into a sterile enamelware sputum cup. A carefully selected bit of sputum was then washed in several changes of sterile salt solution, a portion placed in a sterile mortar and ground up for injection in amounts of 0.05 cc and 0.5 cc diluted with salt solution for injection into mice, the other portion being examined for tubercle bacilli and for the approximate numbers of the other predominating organisms. The peritoneal fluid from the mice was examined after 48 hours or immediately after death by direct typing and by smear for culture on human blood-agar plates. The saliva was obtained after several rinsings with sterile salt solution and before injection it was filtered through sterile cheese cloth. It was injected in amounts of 0.1 cc and 1 cc intraperitoneally into mice, being studied further as in the case of the sputum. The typing of the pneumococci were all carried out with well controlled agglutinating serums tested against known typed organisms. The cultures of pneumococci used in these experiments were supplied by Dr. O. T. Avery of Rockefeller Institute for Medical Research. The determination of pneumococci as differentiated from streptococci was based on the formation of capsule in suitable surroundings and bile solubility, besides the morphology and specific agglutination.

The sputum from 57 definite cases of pulmonary tuberculosis; 29 far advanced cases, all but one open; 24 moderately advanced cases, all open; and 4 incipient cases, two open, were examined in this way by inoculation into mice and the results are given in table 1.

⁴ *Ibid.*, p. 251.

TABLE 1
RESULTS OF THE INJECTION OF WASHED SPUTUM OF CASES OF PULMONARY TUBERCULOSIS INTO MICE

Classification	Tubercle Bacilli		Pneumococci to Field,* Gram Stain				Result in Mouse with 0.05 C U				Result in Mouse with 0.5 C U				Pneumo-coccus Type			Strepto-coccus	Negati-ve Perito-neal Fluid
	+	-	1-10	11-20	21-40	Over 40	Not Fatal	1 Day	1-2 Days	Over 2 Days	Not Fatal	1 Day	1-2 Days	Over 2 Days	II	III	IV		
Incipient 4 cases	1	3	2	1	1	0	4	0	0	0	3	0	1	0	0	0	2	0	2
Moderately advanced, 24 cases (all open cases, one died within one month)	7	1	4	2	1	1	7	0	1	0	6	0	1	1	1	1	1	0	5
Afebrile	9	7	4	6	1	5	12	0	2	2	5	3	7	1	3	2	8	1 Strepto-coccus viridans	2
Far advanced, 29 cases (all open except one, eight died within two months after examinations were made)	18	6	12	5	4	3	20	0	1	3	14	4	6	0	2	3	9	0	10
Afebrile	4	1	0	1	2	2	3	0	1	1	0	1	3	1	0	1	3	1 Hemolytic strepto-coccus with Type IV pneumo-coccus	0
Totals.....	39	18	22	15	9	11	46	0	5	6	28	8	18	3	6	7	23	2	19

* High power magnification.
† No Type I pneumococci were found in this series.
‡ The peritoneal fluid in these cases proved sterile on blood agar plates.

The results noted in table 1 agree well with the results of the previous observations by directly cultivating the washed sputums in pulmonary tuberculosis. The percentage, 33% sputums (57) negative for pneumococci or streptococci by mouse inoculation agree closely with the percentage 37% sputums (216) negative for the same organisms by direct cultivation. It is noteworthy also that a small percentage (4%) of hemolytic streptococcus and streptococcus viridans was found in the sputum while the predominating organisms were pneumococci especially types III and IV, some type II but no type I. There was no relation discoverable between the virulence of the sputums for mice and the condition of the patient.

A few points not brought out in the table may be of interest. In only one case did the results from the injection of 0.05 and of 0.5 c c differ: the first gave hemolytic streptococci, the other pneumococcus IV. In all other cases the two injections checked exactly as to positive results and the type of organism in the peritoneal fluid. In eight of the far advanced cases the peritoneal fluid was sterile after injecting 0.05 c c sputum while mouse which received 0.5 c c gave pneumococcus II in one case and III in one case and IV in six cases. In four of the moderately advanced cases the peritoneal fluid was sterile after injecting the smaller amount of sputum while the 0.5 c c mouse contained pneumococcus II in one case, IV in two cases and Streptococcus viridans in one case.

In order to note what difference, if any, there may be between the organisms harbored in the sputum and saliva of cases of pulmonary tuberculosis and of normal individuals a series of injections into mice were made in the same way as in the previous experiment. The material used came from 21 normal individuals, according to all clinical and laboratory methods available free from abnormal conditions in the respiratory tract and from 10 cases of pulmonary tuberculosis. The results are given in table 2.

Table 2 reveals very little difference between the results with the sputum and saliva of normal individuals, the organisms found being pneumococcus III and IV. The 10 cases of pulmonary tuberculosis examined did not prove entirely satisfactory for comparison with the normal cases, but it is easily apparent that the organisms found in the saliva of cases of pulmonary tuberculosis are identical with those found in the sputum, and as in normal saliva pneumococci III and IV predominate.

In the 21 sputums and salivas from normal individuals nine gave concordant results, while seven revealed pneumococci in the peritoneal fluid of the mice injected with saliva but none in the mice injected with sputum, and five gave

pneumococci in the mice injected with sputum and none in the mice injected with the saliva. Four of the ten cases of pulmonary tuberculosis chosen had given negative results in the previous series (table 1); and in this second series both sputum and saliva were negative. In the ten cases both sputum and saliva gave the same pneumococci in the peritoneal fluid of the injected mice in 3 cases; two gave pneumococci with the saliva and none with the sputum, and two gave pneumococci with the sputum and none with the saliva; the other five were negative with both.

TABLE 2

COMPARISON OF RESULTS OF INJECTION OF SALIVA AND SPUTUM FROM NORMAL INDIVIDUALS AND OPEN CASES OF PULMONARY TUBERCULOSIS INTO MICE

Classification	Specimen	Mouse*				Pneumococcus Type		Negative Peritoneal Fluid
		Not Fatal	Fatal Within			III	IV	
			1 Day	1-2 Days	Over 2 Days			
Normal Individuals 21	Sputum	11	7	3	0	2	12	7
	Saliva	8	9	3	1	2	14	5
Pulmonary Tuberculosis 10 Cases†	Sputum	8	1	1	0	1	4	5
	Saliva	6	2	2	0	1	4	5

* The sputum was injected in amounts of 0.05 cc and 0.5 cc, the saliva in amounts of 0.1 and 1 cc; only the larger results of the injections are recorded.

† Unfortunately four of the cases of pulmonary tuberculosis chosen had previously given negative results in the experiment recorded in table 1 and therefore these results are not valuable for comparison with the results in normal individuals but rather only for comparison of the results with sputum and saliva of the same class.

SUMMARY AND CONCLUSIONS

The organisms from the sputum of open cases of pulmonary tuberculosis obtained by intraperitoneal injection of mice are mainly pneumococci of types III and IV, and a few of type II, the types which are considered saprophytic by Pritchett and Stillman in contradistinction to the pneumococcus I and certain strains of type II. A few hemolytic streptococci and streptococcus viridans were also found in the sputum. As a whole, the organisms found in the sputum from open cases of pulmonary tuberculosis by mouse inoculation differed little from those found in the saliva of the same cases, and in the sputum and saliva of normal individuals.

These results are corroborative of previous results by direct culture methods from which it was concluded that the organisms in the sputum in pulmonary tuberculosis ordinarily play an insignificant part in the pathogenesis of this disease. As far as the presence of these organisms in the lung are concerned they are to be considered more

as would their presence in any nontuberculous disease, and as indicating a condition of lowered local resistance which makes possible a saprophytic residence of such bacteria. Just why the so-called parasitic types are not found remains to be discovered, as does also the infrequent occurrence of pneumococcus pneumonia in pulmonary tuberculosis.

DIPHTHERIA BACILLI FROM POSTOPERATIVE EMPYEMA WOUNDS

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On March 24, 1918, an organism corresponding morphologically to the diphtheria bacillus was found in a culture from a healing post-operative empyema wound. Cases showing this organism increased in number until a short time later sixty of the empyema wounds were infected. The wards were immediately quarantined and all possible care was taken to prevent further spread of the infection. In spite of vigorous treatment positive cultures were obtained after long intervals and in many cases even after 5 months. Because confusing reports have been made of the corynebacteria found in different parts of the body and because of the uncertainty as to the danger attendant with this particular infection an attempt was made to determine the exact nature of the organisms, their morphology, cultural characteristics, virulence for guinea-pigs and specific serum reactions. Efforts were made also to find some satisfactory method of disinfecting the wounds.

Twenty-six strains from infected wounds and five from throats including three positive controls from Hachita, N. M., were isolated in pure culture and studied, with results as follows:

Morphology.—All primary cultures were made on Loeffler's serum, incubated for 18-20 hours at 37 C., and smears stained with Loeffler's alkaline methylene blue and Neisser's method. Wesbrook's classification¹ was followed as a standard; only granular organisms corresponding to types A, C and D were reported as positive, while types A₁, C₁, D₁ and A₂, C₂ and D₂ were noted but disregarded in making routine reports. Practically all positive cultures contained the subtypes in varying numbers and, as found by others, these were present in increasing numbers after the infection had existed for a long period of time. Whether this was due to an inhibition of the granular forms and overgrowth by the solid and barred types, or whether they were involution or "degenerative" forms, is undecided. It was the rule to obtain a number of types together, even from pure cultures which had been carefully picked from single isolated colonies and transferred many times. Once the incubator temperature rose to 42 C. during the night, following which there

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¹ Wesbrook, F. F., Wilson, L. B., and McDaniel, O.: Transactions of the Association of American Physicians, Vol. 25, 1900, p. 198.

was a marked increase in the number of short forms in all cultures. The same phenomenon has been observed by Kolmer and others. All organisms were gram-positive, but easily decolorized. Stained with carbolfuchsin and decolorized with 70% acid alcohol 30 seconds, none were acid fast.

Isolation.—Positive cultures were planted on serum agar, $\frac{1}{2}$ c c horse serum to 5 c c meat infusion agar, and incubated 24 hours. Smears from suspicious colonies were stained and, if positive, one colony was fished and planted on half of a second serum agar plate.

From this second plate, after 24 hours' incubation, colonies positive on smear were planted on Loeffler's serum. These cultures were examined after 18-24 hours' incubation and were accepted only if they were pure Klebs-Loeffler bacilli and showed a few, at least, of Wesbrook's A, C, or D types, but almost invariably the subtypes were present in varying numbers. Broth cultures (meat extract) were made for the inoculation of guinea-pigs from these slants.

Cultural Characteristics.—Although differing in some instances, most observers agree that the true virulent diphtheria bacillus produces fermentation of the monosaccharids dextrose and dextrin; and fails to do so in the case of the disaccharid saccharose.

Fox² states of pseudodiphtheria bacilli that "they are able to ferment dextrose, saccharose and maltose; while true diphtheria bacilli acidify dextrose, dextrin and maltose, but never saccharose." Cary³ concluded "that the monosaccharids are fermentable by true diphtheria bacilli, but that the higher sugars are fermented less characteristically and less uniformly." However, in a few instances, true toxic diphtheria bacilli have been observed which were able to produce acid in saccharose medium and Graham-Smith reported a whole epidemic in 1908 produced by saccharose-fermenting, diphtheria bacilli.

Tubes containing 1% each of saccharose, dextrose, dextrin, galactose, maltose, lactose and glycerol in sugar-free beef-infusion broth were inoculated with pure cultures from each strain. Andrade's solution was used as an indicator and four readings were made at 2-day intervals. If readings had been recorded after a shorter period of time a number of reactions would have been missed, since some of the strains were slower than others in the production of acid. Smears were made and Loeffler's slants inoculated to determine vitality and purity of cultures. Uninoculated control tubes were incubated for the same period of time as the carbohydrates tested. As shown in table 1, the strains examined fell into 6 groups.

The strains in group 1 fermented all the carbohydrates excepting saccharose and glycerol. These strains included cultures from the empyema ward. All of the positive control cultures from clinical cases of pharyngeal diphtheria (Hachita, N. M.) likewise arranged themselves in this group. The virulence of these strains varied considerably; two of the wound cultures, one throat culture (clinical diphtheria, empyema ward) and one control were "very virulent"; one culture from throat of nurse on duty in an empyema ward was of intermediate virulence; one wound culture and one positive control culture from a clinical case (Hachita, N. M.) were of slight virulence. The other positive controls from throat diphtheria were virulent for guinea-pigs. These strains were all recovered at necropsy after which there was no change in their fermentation reactions.

Of the eighteen strains in group 2 fermenting saccharose, dextrose and galactose, and failing to ferment dextrin, maltose, lactose and glycerol, none

² Arch. Int. Med., Sept., 1915.

³ Jour. Inf. Dis., 1917, 20, p. 244.

were of remarkable virulence. Three pigs died 9 days, and one each 15, 16, 20, 22 and 32 days, respectively, after inoculation, and ten are still living. Only one strain was recovered (death after 16 days) but the organism isolated at necropsy showed a change of its fermentation reactions.

None of the strains in groups 3, 4, 5, 6 fermented saccharose or proved virulent for guinea-pigs.

Growth on coagulated blood serum was moist and confluent in all but four strains; 19 were grayish-white in color, two were colorless and ten showed a slight pinkish tinge. All of the virulent strains were moist, confluent, grayish-white and failed to show any proteolytic activity.

TABLE 1
FERMENTATIONS

	Source of Cultures	Virulence for Guinea-Pigs	Saccha-rose	Dex-trose	Galac-tose	Dex-trin	Mal-tose	Lac-tose	Glyc-erol
I	2 wound cultures	} Very virulent	0	+	+	+	+	+	0
	1 throat, empyema								
	1 throat, diphtheric (Hachata, "A")	} Intermediate virulence							
	1 throat, nurse, diphtheric, empyema ward								
	1 wound culture	} Slight virulence							
1 throat, diphtheric (Hachita, "B")									
	1 throat, diphtheric patient "B" control	} Low virulence							
II	8 wound cultures	} Slight virulence Avirulent	+	+	+	0	+	0	0
	10 wound cultures								
III	2 wound cultures	Avirulent	+	÷	+	0	+	0	0
IV	1 wound culture	Avirulent (Xerosis?)	+	+	+	0	+	0	+
V	1 wound culture	Avirulent	+	+	0	0	0	0	+
VI	1 wound culture	Avirulent (Hoagi?)	+	+	0	0	0	0	0

Total strains from empyema ward, 28.

Virulent strains from empyema ward, 5 or 17.8%.

Wound strains from empyema ward, 26.

Virulent wound strains from empyema ward, 3 or 11.5%.

Throat strains from empyema ward, 2.

Virulent throat strains from empyema ward, 2 or 100%.

Controls: Clinical throat strains (Hachita) all saccharose and glycerol negatives, 3. Very virulent, 1; slightly virulent, 1; avirulent, 1.

The formation of a pellicle on broth cultures was not constant and occurred only with the less virulent strains. Observations were made at the end of 24 and 48 hours, using both plain and glucose broth cultures.

For the determination of hemolysis a small loop of culture was mixed in 5 cc of sterile normal salt solution, from which one loopful was added to 5 cc melted agar with 0.5 cc defibrinated horse blood. This was then poured into sterile Petri dishes and incubated. Examination for hemolysis was made with the microscope and was recorded as positive only when the red cell outlines had completely disappeared. Four virulent and two avirulent strains produced a definite zone of hemolysis.

All the strains failed to produce indol in Dunham's peptone medium. Titrations were made on the 4th day.

Seventeen cultures including all the virulent strains gave a scarcely perceptible pink color in litmus milk, while in the remaining fourteen no change was detected.

Virulence.—For Man: In two early cases the patients died; one (throat ++) died March 19, and another who gave positive cultures from wound, nose and throat April 18, died April 20. Four cases developed paralytic conditions even after administration of antitoxin. Unfortunately the cultures from the fatal cases were lost. A nurse developed a typical pharyngeal diphtheria while on duty in the quarantined empyema ward, the organisms from the throat being of intermediate virulence for guinea-pigs.

For Guinea-Pigs: Guinea-pigs weighing less than 300 gm. were inoculated subcutaneously with 0.5% of their body weight, reckoned in cubic centimeters, of a 72-hour broth culture. A control pig was given the same proportional dose, after having had 24 hours previously, 100 units of diphtheria antitoxin intraperitoneally. All animals were examined as soon after death as possible and cultures made from subcutaneous tissues at the site of inoculation, from peritoneal or pleural exudates, when present, and from the heart blood. The control pigs were protected by the diphtheria antitoxin which they received.

Three strains from the empyema ward produced death of guinea-pig in one to two days; two of these strains were isolated from wounds and one from an apparently normal throat. One of the positive control throat cultures (Hachita, N. M.) was also very virulent. The organisms were recovered in all these cases.

One culture, which was obtained from the throat of a nurse 'C' who developed pharyngeal diphtheria while on duty in the quarantined empyema ward, killed a guinea-pig in four days.

Of three wound cultures which produced death of guinea-pig in 6-10 days, two fermented saccharose, the third failed to do so. This was also true of another positive control culture (Hachita, N. M.).

One culture, which produced only a local lesion, was obtained from the throat of a patient who was admitted to the hospital with 'laryngeal diphtheria'. This organism failed to ferment saccharose.

Microscopic sections through the abdominal wall at the site of the inoculation showed ulceration of the surface. The bands of connective tissue in the subcutaneous fatty layer and the fascia were edematous and infiltrated with polymorphonuclear leukocytes and corpuscles. The muscle fibers adjacent to the fascia were infiltrated also. The infection did not penetrate the muscle and the peritoneum was normal. Stained by Weigert's method great quantities of gram-positive bacilli, some stained irregularly, were seen.

Twenty cultures produced no noticeable effect on guinea-pigs. These were all saccharose fermenters.

Specific Serum Reactions.—Tests made to determine the presence of complement fixing, precipitating and agglutinating antibodies in the serum of the infected individuals all failed to give definite results.

Blood cultures were made in all cases with negative results.

In addition, comparative studies were made of cultures from discharging empyema wounds of 30 patients in the base hospital at Camp Travis. None of these cultures showed diphtheria bacilli, but three gave *B. hoffmannii*.

Treatment.—All patients were isolated, quarantined and given diphtheria antitoxin in large amounts when the first positive culture was reported. Schick

tests were not made until after the initial antitoxin inoculations. The reactions were all negative, as one would expect, and when repeated, after an interval of several months, the results were similar. Dichloramin-T solution was used locally but did not prove of value. Later the cases were divided into four groups each of which was treated differently: Group 1 received daily irrigations with Dakin's solution; group 2, wounds were cleaned and swabbed with Mandell's solution daily; group 3, wounds were irrigated daily with diphtheria antitoxin; while the group 4 wounds were cleansed and exposed to direct sunlight as long as possible daily. Cultures made every other day over a period of several months failed to show any marked differences in the results obtained with these various methods of treatment. The decrease in positive cases was slow, and as a rule the carrier state continued until the operative wound was completely healed.

It was noticed that whenever the wounds were curetted, the total number of positive cultures were greatly increased, including many cases which had been considered negative for some time. This probably explains the failure of the therapeutic measures tried as it seems to indicate that many of the diphtheria bacilli were deeply buried between the crevices or within the granulation tissue and for this reason were not reached by the antiseptics used.

SUMMARY

The organisms isolated from empyema wounds were morphologically true diphtheria bacilli.

Of the strains isolated, 17.8% were virulent for guinea-pigs, and all of these strains failed to produce acid when grown in saccharose broth cultures for 8 days. The degree of virulence among saccharose negative strains was variable.

The morphological characteristics presented by both virulent and avirulent strains were the same. All cultures contained a mixture of Westbrook's type A, C and D, with the subtypes.

There was no evidence of the development of specific agglutinins, precipitins or complement fixing substances for diphtheria bacilli (virulent or avirulent) in the serum of the infected individual.

Apparently there was no invasion of the blood stream by the diphtheria bacilli in the wounds.

All methods of treatment tried proved unsatisfactory, due probably to the growth of bacilli deep in the wound granulations. As a rule the carrier state continued until complete healing of the wounds had taken place.

THE INFLUENCE OF ANTIGEN DILUTION ON THE WASSERMANN REACTION

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In a previous report¹ it was shown that higher dilutions of antigens gave stronger positive results in the Wassermann reaction than did lower dilutions of the same antigen. At the present time the results obtained in a more detailed study on the influence of antigen turbidity and antigen dilution will be reported.

TECHNIC

The human serums were heated to about 56 C. for 30 minutes and were glycerolated. All ingredients were so prepared that the test doses were uniform. The test dose of each prepared ingredient was 0.2 cc which gave a total quantity of 1 cc for each test tube.

As complement serum the mixed serums of five guinea-pigs were used in dilutions of 1:5, 1:10 and 1:20.

Three different antigens were used, alcoholic extract of human heart (A. E. H. H.), alcoholic extract of beef heart (A. E. B. H.) and alcoholic extract of rabbit heart (A. E. R. H.). Of each antigen six different portions, A, B, C, D, E and F were prepared. For portion A, 0.1 cc of alcoholic extract was put into a test tube, 0.05 of salt solution was added, the mixture shaken, another 0.05 cc of salt solution was added, the mixture was again shaken and was allowed to stand for about 5 minutes, when quantities of 0.1 cc of physiologic salt solution were added until the mixture amounted to 1 cc, the mixture having been well shaken after each addition of salt solution. In any further dilution of the antigen solution the salt solution was added in quantities of 0.2 cc. Portion A of the antigen solution was used in dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400. For portion B, 0.1 cc of alcoholic extract was put into a test tube, 0.05 cc of salt solution was added, the mixture was shaken, another 0.05 cc of salt solution was added, the mixture was again shaken and was diluted up to 1 cc by the addition of 0.1 cc quantities of salt solution. In further diluting the antigen solution the salt solution was added in quantities of 0.2 cc. Portion B was used in dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400. For portion C 0.1 cc of alcoholic extract was put into a test tube, 0.1 cc of salt solution was added, the mixture was shaken, the dilution was continued by the addition of 0.1 cc quantities of salt solution until the mixture amounted to 1 cc, after which quantities of 0.2 cc were used. Like portions A and B portion C was used in dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400. For portion D 0.1 cc of alcoholic extract was put into a test tube and was diluted by adding 0.2 cc quantities of salt solution. Portion D, like the previous portions, was used in dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400. For portion E 0.1 cc of alcoholic extract was put

into a test tube and was diluted by adding 0.3 cc quantities of salt solution. Portion E was used in dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400. In portion F 0.1 cc of alcoholic extract was diluted by adding 0.4 cc quantities of salt solution. Portion F was used in dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400. Whenever the quantity of salt solution necessary for a certain dilution was not a multiple of the quantities added the proper dilution was obtained by adding the necessary fraction of the regular quantity as, $1 + 0.2 + 0.2 + 0.2 + 0.2 + 0.2 + 0.2 + 0.2 + 1 = 2.5$.

The antihuman hemolytic system was used. Hemolytic amboceptor was used in doses of 1 unit per test tube and the blood corpuscles were used in the form of a 2.5% suspension in salt solution.

Each human serum was tested with each of the 30 different antigen solutions at the same time; complement, blood corpuscles and hemolytic amboceptor having been the same any difference in the results must have been due to difference in the antigen solution. First incubation was in the water-bath at 1 C. for 5 hours followed by 30 minutes in the incubator at 37 C. and second incubation was in the incubator at 37 C. for 1 hour. The results were read and recorded about 2 hours after the sensitized blood corpuscles had been added.

TEST 1

Human serums 1 and 2 from syphilitics under treatment and 3 and 4 from nonsyphilitics were each tested with the 30 different solutions of alcoholic extract of human heart.

TABLE 1

SOLUTIONS OF ALCOHOLIC EXTRACT OF HUMAN HEART OF DIFFERENT DEGREES OF TURBIDITY TESTED IN DILUTIONS VARYING FROM 1:25 TO 1:400

Number of Serum	Kind of Antigen	Portion	Dilution of Antigen	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
1	A. E. H. H.	A	1:25	+	±	0	+	+	±	Strongly positive, 3+ Moderately positive, 2+ Moderately positive, 2+ Weakly positive, 1+ Faintly positive, ±
			1:50	+	+	0	+	+	±	
			1:100	+	+	0	+	+	±	
			1:200	+	+	tr	+	+	±	
			1:400	+	+	±?	+	+	±	
		B	1:25	+	0	0	+	+	±	Strongly positive, 5+ Strongly positive, 5+ Strongly positive, 4+ Moderately positive, 2+ Weakly positive, 1+
			1:50	+	0	0	+	+	±	
			1:100	+	tr	0	+	+	±	
			1:200	+	+	0	+	+	±	
			1:400	+	+	tr	+	+	±	
		C	1:25	+	0	0	+	+	±	Strongly positive, 5+ Strongly positive, 6+ Strongly positive, 5+ Strongly positive, 3+ Moderately positive, 2+
			1:50	±	0	0	+	+	±	
			1:100	+	0	0	+	+	±	
			1:200	+	±	0	+	+	±	
			1:400	+	+	0	+	+	±	
		D	1:25	+	+	0	+	+	±	Moderately positive, 2+ Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 4+ Moderately positive, 2+
			1:50	+	±	0	+	+	±	
			1:100	+	tr	0	+	+	±	
			1:200	+	tr	0	+	+	±	
			1:400	+	+	0	+	+	±	

* Explanation: In all tables + means complete hemolysis; ±, hemolysis between 50 and 100%; tr (trace) hemolysis up to 50%; 0, no hemolysis.

Serums 3 and 4, tested exactly in the same way as serums 1 and 2, gave uniformly negative results.

TABLE 1—Continued

SOLUTIONS OF ALCOHOLIC EXTRACT OF HUMAN HEART OF DIFFERENT DEGREES OF TURBIDITY
TESTED IN DILUTIONS VARYING FROM 1:25 TO 1:400

Number of Serum	Kind of Antigen	Por- tion	Dilution of Antigen	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
2	A. E. H. H.	E	1:25	+	+	tr	+	+	±	Weakly positive, 1+
			1:50	+	+	0	+	+	±	Moderately positive, 2+
			1:100	+	±	0	+	+	±	Strongly positive, 3+
			1:200	+	±	0	+	+	±	Strongly positive, 3+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		F	1:25	+	+	±?	+	+	±	Faintly positive, ±
			1:50	+	+	tr	+	+	±	Weakly positive, 1+
			1:100	+	+	0	+	+	±	Moderately positive, 2+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		A	1:25	+	tr	0	+	+	±	Strongly positive, 4+
			1:50	+	tr	0	+	+	±	Strongly positive, 4+
			1:100	+	±	0	+	+	±	Strongly positive, 3+
			1:200	+	+	tr	+	+	±	Weakly positive, 1+
			1:400	+	+	±?	+	+	±	Faintly positive, ±
		B	1:25	+	±	0	+	+	±	Strongly positive, 3+
			1:50	+	tr	0	+	+	±	Strongly positive, 4+
			1:100	+	±	0	+	+	±	Strongly positive, 3+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	tr	+	+	±	Weakly positive, 1+
		C	1:25	+	±	0	+	+	±	Strongly positive, 3+
			1:50	+	0	0	+	+	±	Strongly positive, 5+
			1:100	+	0	0	+	+	±	Strongly positive, 5+
			1:200	+	±	0	+	+	±	Strongly positive, 3+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		D	1:25	+	+	±	+	+	±	Negative, —
			1:50	+	+	tr	+	+	±	Weakly positive, 1+
			1:100	+	+	0	+	+	±	Moderately positive, 2+
			1:200	+	±	0	+	+	±	Strongly positive, 3+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		E	1:25	+	+	±	+	+	±	Negative, —
			1:50	+	+	±?	+	+	±	Faintly positive, ±
			1:100	+	+	tr	+	+	±	Weakly positive, 1+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		F	1:25	+	+	±	+	+	±	Negative, —
			1:50	+	+	±	+	+	±	Negative, —
			1:100	+	+	±?	+	+	±	Faintly positive, ±
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	0	+	+	±	Moderately positive, 2+

The results obtained by testing the 30 different solutions of the same alcoholic extract of human heart are shown in table 1. Portion C gave the strongest positive results with the syphilitic serums and the strongest positive results were obtained with the dilution of 1:50. In portions A and B the stronger results were obtained with lower dilutions of antigen and with portions D, E and F the higher dilutions, such as 1:100 and 1:200, gave stronger positive results than did the lower dilutions. The serums from nonsyphilitic persons gave negative results throughout.

TEST 2

With serums 5 and 6 from known syphilitics under treatment and 7 and 8 from nonsyphilitic persons each of the six different portions, A, B, C, D, E and F of alcoholic extract of beef heart was tested in 5 different dilutions, dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400.

TABLE 2
SOLUTIONS OF ALCOHOLIC EXTRACT OF BEEF HEART OF DIFFERENT DEGREES OF TURBIDITY
TESTED IN DILUTIONS VARYING FROM 1:25 TO 1:400

Number of Serum	Kind of Antigen	Portion	Dilution of Antigen	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
5	A. E. B. H.	A	1:25	±	0	0	+	+	±	Strongly positive,	6+
			1:50	tr	0	0	+	+	±	Strongly positive,	8+
			1:100	±	0	0	+	+	±	Strongly positive,	6+
			1:200	+	±	0	+	+	±	Strongly positive,	3+
			1:400	+	+	0	+	+	±	Moderately positive,	2+
		B	1:25	+	0	0	+	+	±	Strongly positive,	5+
			1:50	tr	0	0	+	+	±	Strongly positive,	8+
			1:100	+	0	0	+	+	±	Strongly positive,	5+
			1:200	+	0	0	+	+	±	Strongly positive,	5+
			1:400	+	±	0	+	+	±	Strongly positive,	3+
		C	1:25	+	tr	0	+	+	±	Strongly positive,	4+
			1:50	+	0	0	+	+	±	Strongly positive,	5+
			1:100	±	0	0	+	+	±	Strongly positive,	6+
			1:200	+	tr	0	+	+	±	Strongly positive,	4+
			1:400	+	tr	0	+	+	±	Strongly positive,	4+
		D	1:25	+	+	tr	+	+	±	Weakly positive,	1+
			1:50	+	+	tr	+	+	±	Weakly positive,	1+
			1:100	+	+	0	+	+	±	Moderately positive,	2+
			1:200	+	±	0	+	+	±	Strongly positive,	3+
			1:400	+	±	0	+	+	±	Strongly positive,	3+
		E	1:25	+	+	tr	+	+	±	Weakly positive,	1+
			1:50	+	+	tr	+	+	±	Weakly positive,	1+
			1:100	+	+	0	+	+	±	Moderately positive,	2+
			1:200	+	±	0	+	+	±	Strongly positive,	3+
1:400	+		±	0	+	+	±	Strongly positive,	3+		
F	1:25	+	+	tr	+	+	±	Weakly positive,	1+		
	1:50	+	+	tr	+	+	±	Weakly positive,	1+		
	1:100	+	+	tr	+	+	±	Weakly positive,	1+		
	1:200	+	+	0	+	+	±	Moderately positive,	2+		
	1:400	+	+	0	+	+	±	Moderately positive,	2+		
6	A. E. B. H.	A	1:25	+	tr	0	+	+	±	Strongly positive,	4+
			1:50	+	0	0	+	+	±	Strongly positive,	5+
			1:100	+	tr	0	+	+	±	Strongly positive,	4+
			1:200	+	+	0	+	+	±	Moderately positive,	2+
			1:400	+	+	tr	+	+	±	Weakly positive,	1+
		B	1:25	+	tr	0	+	+	±	Strongly positive,	4+
			1:50	+	0	0	+	+	±	Strongly positive,	5+
			1:100	+	0	0	+	+	±	Strongly positive,	5+
			1:200	+	±	0	+	+	±	Strongly positive,	3+
			1:400	+	+	0	+	+	±	Moderately positive,	2+
		C	1:25	+	+	tr	+	+	±	Weakly positive,	1+
			1:50	+	+	0	+	+	±	Moderately positive,	2+
			1:100	+	±	0	+	+	±	Strongly positive,	3+
			1:200	+	±	0	+	+	±	Strongly positive,	3+
			1:400	+	+	0	+	+	±	Moderately positive,	2+

Serums 7 and 8, nonsyphilitic, tested in the same way as serums 5 and 6, gave uniformly negative results.

TABLE 2—Continued

SOLUTIONS OF ALCOHOLIC EXTRACT OF BEEF HEART OF DIFFERENT DEGREES OF TURBIDITY
TESTED IN DILUTIONS VARYING FROM 1: 25 TO 1: 400

Number of Serum	Kind of Antigen	Portion	Dilution of Antigen	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
	D	1:25	+	+	± [?]	+	+	±	Faintly positive,	±
		1:50	+	+	± [?]	+	+	±	Faintly positive,	±
		1:100	+	+	tr	+	+	±	Weakly positive,	1+
		1:200	+	+	0	+	+	±	Moderately positive,	2+
		1:400	+	+	0	+	+	±	Moderately positive,	2+
	E	1:25	+	+	± [?]	+	+	±	Negative,	—
		1:50	+	+	± [?]	+	+	±	Faintly positive,	±
		1:100	+	+	± [?]	+	+	±	Faintly positive,	±
		1:200	+	+	tr	+	+	±	Weakly positive,	1+
		1:400	+	+	0	+	+	±	Moderately positive,	2+
	F	1:25	+	+	± [?]	+	+	±	Negative,	—
		1:50	+	+	± [?]	+	+	±	Negative,	—
		1:100	+	+	± [?]	+	+	±	Faintly positive,	±
		1:200	+	+	tr	+	+	±	Weakly positive,	1+
		1:400	+	+	0	+	+	±	Moderately positive,	2+

Table 2 shows the results obtained with the 30 different solutions of alcoholic extract of beef heart. Portions A and B gave the strongest positive results. In portions A and B the dilution of 1: 50 gave the strongest positive results, in portion C 1: 100 gave the strongest positive results and of portions D, E and F the dilutions of 1: 200 and 1: 400 gave stronger positive results than did dilutions of 1: 25, 1: 50 and 1: 100. Although dilutions of 1: 200 and 1: 400 of portions D, E and F gave stronger positive results than did the lower dilutions of the same portions these results were greatly inferior to those obtained with portions A and B in dilutions of 1: 50. With the serums from nonsyphilitic persons negative results were obtained throughout.

TEST 3

The 30 different solutions of alcoholic extract of rabbit heart were tested on two serums from known syphilitics and two from nonsyphilitic persons, serums Nos. 9, 10, 11 and 12.

TABLE 3

SOLUTIONS OF ALCOHOLIC EXTRACT OF RABBIT HEART OF DIFFERENT DEGREES OF TURBIDITY
TESTED IN DILUTIONS VARYING FROM 1: 25 TO 1: 400

Number of Serum	Kind of Antigen	Portion	Dilution of Antigen	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
9	A. E. R. H.	A	1:25	+	+	tr	+	+	±	Weakly positive, 1+
			1:50	+	+	0	+	+	±	Moderately positive, 2+
			1:100	+	±	0	+	+	±	Strongly positive, 3+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	tr	+	+	±	Weakly positive, 1+

Serums 11 and 12, nonsyphilitic, tested in the same way as serums 9 and 10, gave uniformly negative results.

TABLE 3—Continued

SOLUTIONS OF ALCOHOLIC EXTRACT OF RABBIT HEART OF DIFFERENT DEGREES OF TURBIDITY
TESTED IN DILUTIONS VARYING FROM 1:25 TO 1:400

Number of Serum	Kind of Antigen	Por- tion	Dilution of Antigen	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
10	A. E. R. H.	B	1:25	+	+	0	+	+	±	Moderately positive, 2+
			1:50	+	±	0	+	+	±	Strongly positive, 3+
			1:100	+	±	0	+	+	±	Strongly positive, 3+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	tr	+	+	±	Weakly positive, 1+
		C	1:25	+	+	tr	+	+	±	Weakly positive, 1+
			1:50	+	±	0	+	+	±	Strongly positive, 3+
			1:100	+	tr	0	+	+	±	Strongly positive, 4+
			1:200	+	±	0	+	+	±	Strongly positive, 3+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		D	1:25	+	+	±	+	+	±	Negative, —
			1:50	+	+	±?	+	+	±	Faintly positive, ±
			1:100	+	+	tr	+	+	±	Weakly positive, 1+
			1:200	+	+	tr	+	+	±	Weakly positive, 1+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		E	1:25	+	+	±	+	+	±	Negative, —
			1:50	+	+	±	+	+	±	Negative, —
			1:100	+	+	±?	+	+	±	Faintly positive, ±
			1:200	+	+	tr	+	+	±	Weakly positive, 1+
			1:400	+	+	tr	+	+	±	Weakly positive, 1+
		F	1:25	+	+	±	+	+	±	Negative, —
			1:50	+	+	±	+	+	±	Negative, —
			1:100	+	+	±	+	+	±	Negative, —
			1:200	+	+	tr	+	+	±	Weakly positive, 1+
			1:400	+	+	tr	+	+	±	Weakly positive, 1+
		A	1:25	+	+	0	+	+	±	Moderately positive, 2+
			1:50	+	±	0	+	+	±	Strongly positive, 3+
			1:100	+	+	0	+	+	±	Moderately positive, 2+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	tr	+	+	±	Weakly positive, 1+
		B	1:25	+	+	0	+	+	±	Moderately positive, 2+
			1:50	+	+	0	+	+	±	Moderately positive, 2+
			1:100	+	+	0	+	+	±	Moderately positive, 2+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	tr	+	+	±	Weakly positive, 1+
		C	1:25	+	+	tr	+	+	±	Weakly positive, 1+
			1:50	+	±	0	+	+	±	Strongly positive, 3+
			1:100	+	tr	0	+	+	±	Strongly positive, 4+
			1:200	+	±	0	+	+	±	Strongly positive, 3+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		D	1:25	+	+	±?	+	+	±	Faintly positive, ±
			1:50	+	+	±?	+	+	±	Faintly positive, ±
			1:100	+	+	tr	+	+	±	Weakly positive, 1+
			1:200	+	+	0	+	+	±	Moderately positive, 2+
			1:400	+	+	0	+	+	±	Moderately positive, 2+
		E	1:25	+	+	±	+	+	±	Negative, —
			1:50	+	+	±?	+	+	±	Faintly positive, ±
			1:100	+	+	±?	+	+	±	Faintly positive, ±
1:200	+		+	tr	+	+	±	Weakly positive, 1+		
1:400	+		+	tr	+	+	±	Weakly positive, 1+		
F	1:25	+	+	±	+	+	±	Negative, —		
	1:50	+	+	±	+	+	±	Negative, —		
	1:100	+	+	±	+	+	±	Negative, —		
	1:200	+	+	±?	+	+	±	Faintly positive, ±		
	1:400	+	+	tr	+	+	±	Weakly positive, 1+		

Table 3 shows the results obtained with the 30 different solutions of alcoholic extract of rabbit heart on two serums giving positive results and two serums giving negative results. Portion C gave the strongest positive results, and the strongest results of all was obtained with portion C diluted 1:100. Of portions A and B, the strongest results were obtained with dilutions of 1:50 and 1:100, while portions D, E and F gave their strongest results in the higher dilutions. On the serums from the nonsyphilitic persons negative results were obtained throughout.

SUMMARY

The turbidity of the antigen solution and the dilution of the antigen solution greatly influenced the results obtained with the Wassermann reaction with serums from syphilitic persons.

For each antigen there seems to be an optimum turbidity and an optimum dilution. Antigen diluted too slowly or too rapidly lost in antigenic value.

The optimum dose of antigen would be the proper dose to use in the Wassermann reaction. The optimum dilution of antigen may be somewhat higher than is commonly used; alcoholic extract of human heart gave the strongest positive results in a dilution of 1:50 and alcoholic extract of beef heart and of rabbit heart gave the strongest positive results in dilutions of 1:100.

When the antigen was diluted too slowly the optimum dilution of that particular solution was low, and when the antigen was diluted too rapidly the optimum dilution of that particular solution was high; but neither was equal in antigenic value to the optimum dilution of the optimum turbidity.

With serums from nonsyphilitic persons the results were uniformly negative.

STUDIES IN THE NITROGEN METABOLISM OF BACTERIA

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The experiments here recorded were undertaken with the idea of gaining some information, if possible, as to the materials which usually serve as a source of nitrogen to bacteria, the actual form in which the nitrogenous substances are absorbed, and whether all simple dialyzable protein decomposition products are of equal usefulness in building up the cell substance.

The bulk of investigations of proteolysis in the past has had to do with the identification or determination of end-products. The early researches using mixed cultures on putrescible materials, although not usually controlled as to the exact chemical constituents of the medium, or the strains of bacteria employed, showed that the formation of certain substances such as ammonia, hydrogen sulphid, skatol, and so forth is characteristic of putrefaction irrespective of the precise nature of the protein or the particular varieties of organisms present. Productive of much more knowledge of actual bacterial metabolism was the long list of researches using pure cultures and mediums of known composition, making tests to determine the nature of the compounds formed. The indol test is the best known and most useful result of the investigations carried out on peptones or other tryptophane-containing medium.

Herter and Broeck¹ using a 1% preparation of casein with *B. proteus* demonstrated the presence of primary amines, hydrogen sulphid, fatty acids, aromatic oxyacids, indol, and indol acetic acid. Nawiasky² compared the products used up by *B. proteus* on a medium rich in peptone with those assimilated by *Vibrio Finkler*, *B. fecalis-alkaligenes*, and *B. mesentericus*, and states that *B. proteus* attacks albuminoses much more actively than do the others, but that its action on peptones and creatin was less marked. Rettger³ planted *B. putrificus* Bienstock, *B. edematis maligni*, *B. anthracis-symptomatici*, *B. coli*, and *B. lactis-aerogenes* on special egg-meat medium and tested for indol, skatol, phenol, aromatic oxyacids, skatol-carbonic acid, tyrosin, leucin, albumoses, peptones, tryptophane, hydrogen sulphid, and mercaptan, his con-

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¹ Jour. Biol. Chem., 1911, 9, p. 491.

² Arch. f. Hyg., 1908, 64, p. 33.

³ Jour. Biol. Chem., 1906, 2, p. 71.

clusion being that only the obligate anaerobes cause true putrefaction. Later with Newell⁴ he tried 26 strains of *B. proteus* on egg-meat medium, blood fibrin suspended in broth, and Uschinsky's medium and concluded that, while actively proteolytic, *B. proteus* does not form those end-products characteristic of putrefaction in the narrow sense of the word. Effront⁵ studied *B. proteus*, *B. sporogenes*, *B. mesentericus*, *B. butyricus*, *B. putrificus* Bienstock, etc., in a similar manner on albuminoid mediums. Sasaki's⁶ researches on the particular substances formed from glycyl-glycin, glycyl tyrosin, cystin and tyrosin are of interest and value as pictures of a certain stage of protein degradation, as are the works of Brasch⁷ on glutamic acid, serin, and tyrosin.

While investigations like those mentioned are of diagnostic importance, and throw much light on the types of chemical activity of which a given bacterium is capable, a definite idea of the metabolism of an organism cannot be obtained from purely qualitative data or from a single quantitative determination of some particular product. For such purposes a series of quantitative determinations should be made on a favorable medium, under conditions of vigorous normal growth. Many factors enter into the nature and amount of decomposition products formed: first, the availability of the nitrogenous compounds present, depending on whether or not the organism in question is capable of elaborating a ferment to digest them, the molecular point of attack of the enzyme, whether intermediate products are present, and, if so, what and in what quantity, what proportion of the split molecules are resynthesized into bacterial protein and which are left in the medium, and whether or not some of the compounds present are assimilated in preference to others. In the case of any particular end-product it is desirable to know whether it represents a portion of the protein molecule unattacked by the bacteria, a true cell excretion, or something set free by the death of the cell or by autolysis. Any systematic investigation of the proteolytic activity of an organism should include at least the following points:

1. Determination of the amounts present of certain available food substances.
2. Periodic determination of the amounts of one or more decomposition products used as a measure of proteolysis.
3. Allowances for such factors as rate of increase in numbers, presence of other non-nitrogenous foodstuffs, presence of decomposi-

⁴ Jour. Biol. Chem., 1912, 13, p. 341.

⁵ Monit. Scient. Quesneville, 1911, I, p. 489.

⁶ Biochem. Ztschr., 1912, 41, p. 174; 1912, 47, pp. 462-472; 1914, 59, p. 429; Sasaki, Takaoki and Otsuka: Biochem. Ztschr., 1912, 39, p. 208.

⁷ Biochem. Ztschr., 1909, 18, p. 380; 1909, 22, p. 403.

tion products toxic to the cells or inhibitory of their activity, and products of autolysis of dead cells.

The infinite complexity of proteins, their delicate susceptibility to change under the influence of chemical and physical agents used for their isolation and purification, and the purely technical difficulties involved in an attempt to avoid accidental contaminations while preserving optimum conditions of growth as well as the refinements of chemical technic necessary to quantitative determination, have tended to discourage this type of research, with the result that there are comparatively few such studies on record, all more or less incomplete.

Among the end-products of putrefaction which have been determined quantitatively as a measure of proteolysis, ammonia has been the most common. Effront⁸ made use of it in his studies on yeast. Emmerling and Reiser⁹ stated that at least 25% of the nitrogen in gelatin was converted into ammonia by *B. fluorescens-liquefaciens*, while Arnaud and Charrin¹⁰ found that in three days *B. pyocyaneus* converted 91% of the nitrogen in asparagin into ammonia and that a 3 weeks' culture in gelatin contained ammonia to the amount of 70% of the total nitrogen. Boencke¹¹ and Kendall and Farmer¹² determined ammonia to check the degree of proteolysis in their studies on the protein-sparing influence of carbohydrate. While the information thus obtained is valuable, particularly in the case of the putrefactive saprophytes, the ammonia curve admits of varying interpretations unless checked by other determinations. Moreover, many pathogens do not liberate much ammonia during their short period of life in artificial cultures (Berghaus¹³). The quantitative determination of indol and of phenol, valuable as they are in the study of *B. coli* and similar organisms, has the same limitation.

At the other extreme of the metabolic process, the progress of proteolysis may be observed by systematic determinations of the amount of coagulable protein remaining in the culture medium. De Waele and Vendevelde¹⁴ worked out these results with a variety of organisms planted on gelatin, milk, and casein broth in an endeavor to settle the question of the specificity of bacterial proteases. Bainbridge,¹⁵ Rettger and Sperry¹⁶ working with representative pathogenic and saprophytic strains on medium composed of mineral salts and pure protein (crystallized egg albumin, serum protein, alkali albumin, and edestin) have proven that such growth as may occur on solutions of unchanged proteins is due to the minute amounts of nitrogenous impurities present, and that in the absence of split-protein products the germs are unable to attack native albumin.

⁸ Compt. rend. Acad. de sc., 1908, 146, p. 779.

⁹ Ber. d. deutsch. chem. Gesellsch., 1902, Part 3, p. 700.

¹⁰ Compt. rend. Acad. de sc., 1891, 112, p. 755.

¹¹ Arch. f. Hyg., 1911, 74, p. 81.

¹² Jour. Biol. Chem., 1912, 12, p. 13; 1912, 13, p. 63.

¹³ Arch. f. Hyg., 1908, 64, p. 1.

¹⁴ Centralbl. f. Bakteriöl., I. O., 1903, 39, p. 353.

¹⁵ Jour. Hyg., 1911, II, p. 341.

¹⁶ Am. Jour. Physiol., 1902, 8, p. 284.

Sperry: Report at Soc. Am. Bact., Montreal, Dec. 31, 1913.

Sperry and Rettger: Jour. Biol. Chem., 1914, 20, p. 445.

The quantitative determination of amino-acids is particularly useful, especially if checked by comparison with the amount of destruction of the protein or split-protein which is their source, or, in experiments using the amino-acids as a source of nitrogenous food, estimations of the ammonia which is their fate (Nawiasky¹⁷). Until very recently Sørensen's formol-titration method¹⁸ has been the most in use. This procedure was followed by Rosenthal and Patai¹⁹ who planted virulent and avirulent strains of staphylococcus, streptococcus and *B. coli* on broth. With all three of these organisms they observed an initial sharp rise of amino-acid, followed by a more gradual rate of increase. Meserintsky²⁰ observed a progressive augmentation of the amount of amino-acid present in a 10% gelatin culture of *B. prodigiosus*, which, however, represented only a portion of the non-coagulable nitrogenous material present. Frouin and Ledebt,²¹ working with *B. coli*, *B. typhosus*, *B. dysenteriae* and *V. cholerae* on a solution of non-nitrogenous mineral salts enriched with amino-acids were unable to detect any decrease during the first 24 hours of growth, though the diminution became apparent later. Kendall, Day and Walker²² attempted to use Sørensen technic in addition to determinations of ammonia, total nitrogen and acidity in their work on the protein-sparing action of glucose, but abandoned the amino-acid estimation because "the results furnished no information of importance."

The more delicate and accurate method of Van Slyke²³ now makes it possible to obtain much more significant estimation of the progress of proteolysis, since the micro-apparatus is accurate to 0.005 milligram of nitrogen.

This method was used by Sears²⁴ on peptone, broth, and gelatin cultures of a great variety of organisms. He also determined ammonia and creatinin. While some of his cultures showed a gradual increase in amino-nitrogen, the majority exhibited fluctuating rises and falls indicating in his opinion that amino-acids were formed and broken continuously during the life of the culture.

To arrive at a more nearly complete understanding of the nitrogen metabolism of bacteria it would seem valuable not only to apply such a series of tests to growth on peptone-containing mediums, but also to study the amino-acid content of cultures on some material which contains native albumin. This is particularly true of the pathogens, many of which do not thrive on plain peptone or broth. Moreover, their behavior in a medium closely resembling blood-serum or some other body fluid is well worth detailed consideration, both for the insight it might give into the process of infection, and the possibility of the usefulness of such data in attacking the chemical side of problems of immunity. Ascitic fluid is more similar to blood serum than any other material which can easily be obtained sterile in sufficient quantity for such purposes. Accordingly, determinations have been made on *B. typhosus*, *B. proteus*, *B. pyocyaneus*, and staphylococcus grown on broth, on pure ascitic fluid, and on mixtures of equal quantities of the two. In order to rule out the protein-sparing properties of carbohydrates as much as possible in the interests of simplicity, sugar-free broth was used. Such broth may

¹⁷ Arch. f. Hyg., 1908, 66, p. 209.

¹⁸ Biochem. Ztschr., 1907, 7, pp. 45 and 407.

¹⁹ Centralbl. f. Bakteriol., I, O., 1914, 73, p. 406.

²⁰ Biochem. Ztschr., 1910, 29, p. 104.

²¹ Compt. rend. Soc. de biol., 1911, 70, p. 24.

²² Jour. Am. Chem. Soc., 1913, 35, p. 1201.

²³ Jour. Biol. Chem., 1911, 9, p. 185; 1912, 12, p. 275; 1913, 16, p. 121; 1915, 23, p. 407.

²⁴ Jour. Infect. Dis., 1916, 19, p. 105.

contain considerable amounts of amino-acid determined by Van Slyke's method in addition to the peptones, but no coagulable albumin or carbohydrates. In ascitic fluid, on the other hand, the amount of amino-acid and other protein decomposition products is small, while the nitrogen present as coagulable protein is considerable. A small amount of carbohydrate is probably present, but no tests for it were made. A mixture of equal parts of broth and ascitic fluid represents a middle ground between these conditions. In the experiments recorded in tables 4 and 10 ascitic fluid was distributed in equal amounts (10 cc) in a series of sterile test tubes, and each inoculated with a loopful of broth culture. At regular intervals the amino-acid content of one tube was determined, the loss by evaporation being carefully made up with distilled water. While this method has the advantage of permitting determinations to be made without danger of contamination of the material destined for later analysis, there is always the possibility of a considerable variation in the seeding of the different tubes or even in the samples of ascitic fluid. The rest of the work was done with flasks containing a quarter of a liter or more of the medium. Tight fitting rubber caps over the cotton stoppers protected against evaporation. At intervals samples were removed with sterile pipettes after shaking the flask to render the medium homogeneous. These were centrifuged and the supernatant liquid (practically, though probably not absolutely, free of bacterial cells) was tested for the amount of amino-acid present, and in some cases for coagulable protein. In cases where numbers of bacteria present are given, they were determined by counting agar plates made with the customary quantitative precautions. The Kjeldahl determinations were run in duplicate, and the results averaged if they did not check but showed a difference of less than 0.5 cc in the titration. Figures showing a wide variation were discarded. Two Van Slyke determinations were made routinely, checked by a third in case the amounts of nitrogen liberated did not agree within 0.03 cc.

TABLE 1
STAPHYLOCOCCUS

Incubation	Milligrams N As Amino-Acid per C C in Broth	
	Flask 1 (2% Peptone)	Flask 2 (1.5% Peptone)
Sterile.....	0.606	0.407
1 day.....	0.438	0.333
2 days.....	0.315
3 days.....	0.280
5 days.....	0.280	0.319
7 days.....	0.390	0.328
9 days.....	0.332	0.376
11 days.....	0.369
14 days.....	0.252	
19 days.....	0.380	

The staphylococcus cultures on broth (table 1, chart 1a) show a drop to 0.29 mg. though flask 1 originally contained nearly half again as much amino-acid as flask 2. Flask 1 on the chart is shown as having reached this minimum two days later than flask 2 but this may well be due to the fact that no 3-day determination was recorded. In flask 2 there was a steady gradual rise after reaching the minimum until the point when it had to be discarded on account of contamination. Flask 1 shows fluctuations which are without significance when not checked by determinations of other substances.

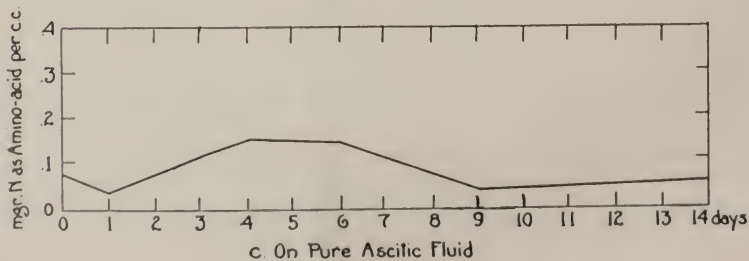
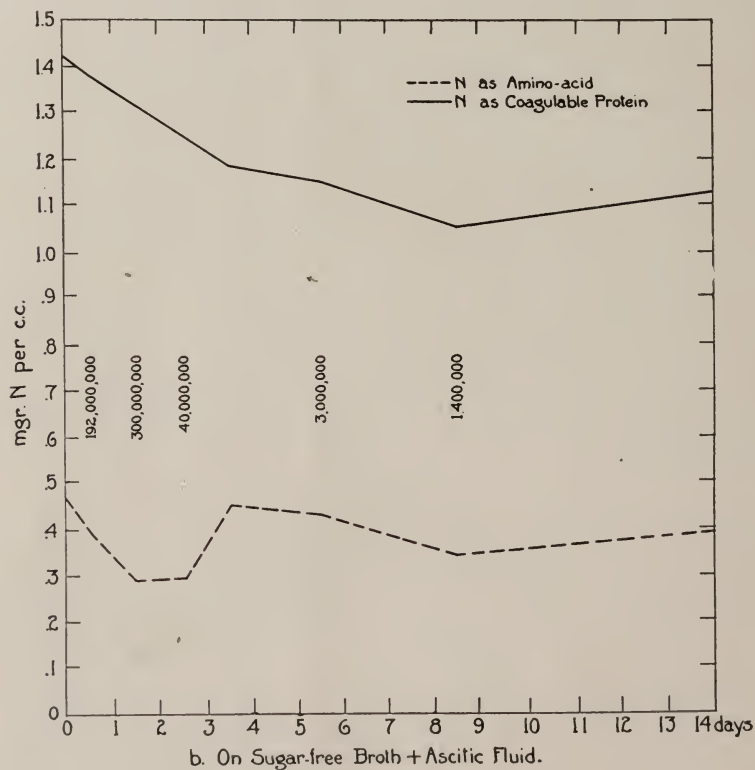
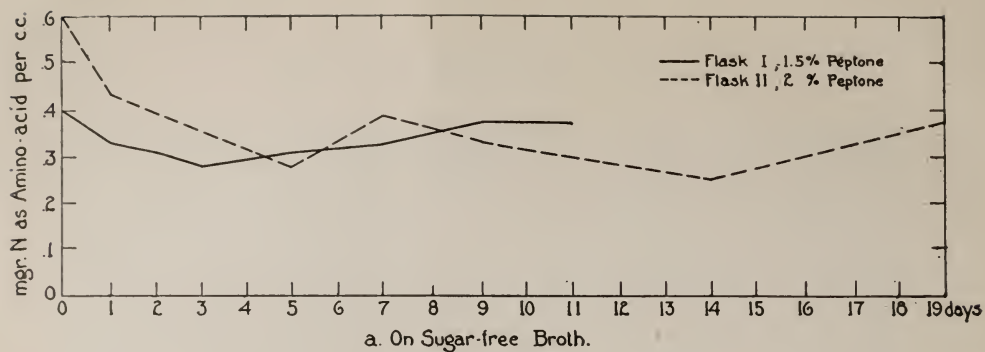


CHART I. STAPHYLOCOCCUS.

On a mixture of broth and ascitic fluid (table 2 and chart 1 b) the amino-acid curve shows a decided fall during the period of most rapid multiplication, followed by a rise and later some fluctuation.

TABLE 2
STAPHYLOCOCCUS

Milligrams N per C C As Amino-Acid and As Coagulable Protein in a Mixture of Ascitic Fluid and Broth			
Incubation	Amino-Acid	Coagulable Protein	Bacteria per C C
Sterile	0.468	1.420	
12 hours	0.393	1.382	192,000,000
36 hours	0.295	1.312	300,000,000
60 hours	0.306	40,000,000
84 hours	0.445	1.189	
132 hours	0.425	1.158	3,000,000
8½ days	0.341	1.052	1,400,000
14 days	0.390	1.134	

On pure ascitic fluid (table 3, chart 1 c) although the amount of amino-acid present to begin with was much smaller than in any of the other flasks, we observe the same initial drop followed by a slight rise and a subsequent fall.

TABLE 3
STAPHYLOCOCCUS

Milligrams N As Amino-Acid per C C of Pure Ascitic Fluid							
Incubation.....	Sterile	1 day	3 days	4 days	6 days	9 days	14 days
Milligrams N.....	0.067	0.083	0.112	0.145	0.143	0.033	0.054

Apparently this organism readily assimilates a part at least of the amino-acids found in each of these mediums but attacks the coagulable protein to some extent from the first. After about 3 days there comes a period when amino-acids accumulate, apparently due partly to autolysis and partly to the fact that the original content of assimilable amino-acid is exhausted, and only a portion of those liberated from the proteins, peptones, etc., can be used by the organism. The increase of coagulable protein after the 8th day may be due to the fact that, as the cultures grew older, it became increasingly difficult to get a clear supernatant fluid by centrifugation.

The results obtained in similar mediums with *B. pyocyaneus* are noticeably different. On pure ascitic fluid (table 4, chart 2 c), after an initial drop, a steady rapid rise in amino-acid is observed.

TABLE 4
B. PYOCYANEUS

Milligrams N As Amino-Acid per C C of Ascitic Fluid								
Incubation.....	Sterile	1 day	2 days	3 days	5 days	8 days	10 days	12 days
Milligrams N.....	0.077	0.049	0.013	0.074	0.099	0.249	0.493	0.695

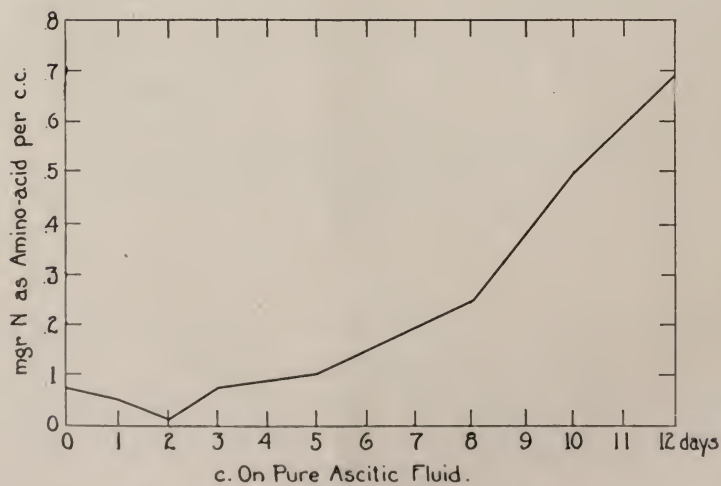
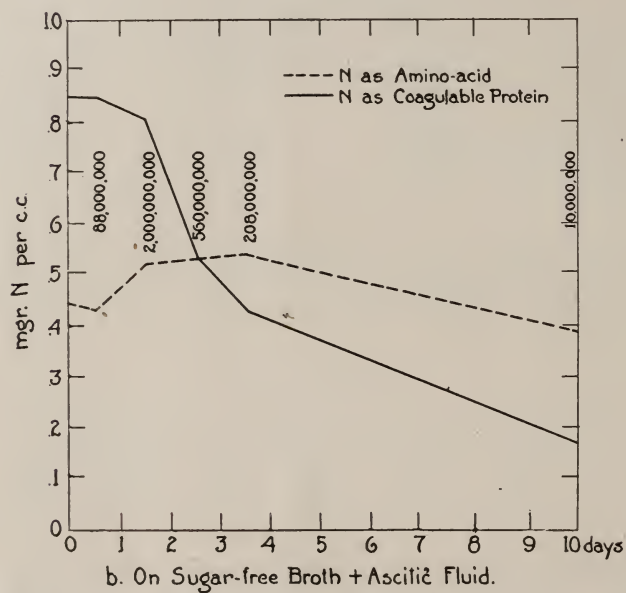
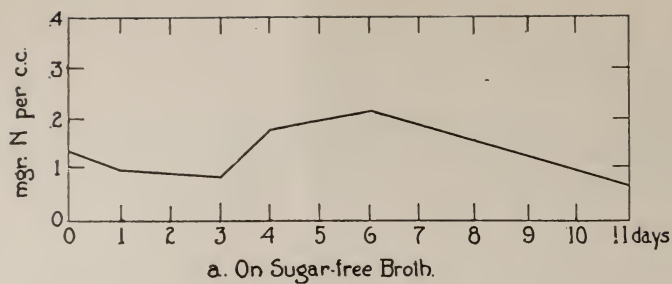


Chart 11. BACILLUS PROCYANEUS.

On sugar-free broth, however, there is a variation (table 5, chart 2 a) which may be significant. After the original drop (which would perhaps be more noticeable if a 2-day determination had been made) and a subsequent increase between the 3rd and 6th days, there is an unmistakable drop shown on the 11th day. Apparently, after exhausting the favorite amino-acids present in the peptone, the organism attacks the previously unused amino-acid while in an abundance of available protein it continues uneconomically to select for synthesis the molecules best adapted to its needs.

TABLE 5
B. PYOCYANEUS

Milligrams N As Amino-Acid per C C of Broth						
Incubation.....	Sterile	1 day	3 days	4 days	6 days	11 days
Milligrams N.....	0.1384	0.098	0.086	0.184	0.215	0.066

On a mixture of broth and ascitic fluid (table 6, chart 2 b) the results are somewhat similar, except that the rise in amino-acid occurred earlier. Here, too, we have a decrease in amino-nitrogen after the fourth day, although there is destruction of protein going on at the same time. Curiously enough, the greatest destruction of protein took place after the maximum count had been passed and at a time when the free amino-acids were not noticeably increasing. The protein destruction might be attributed to the activity of the enzymes previously secreted by the bacteria but in that case an increase of amino-acid would be expected. The fact that the acids do not remain free in the culture can be explained on either of two suppositions: that the enzymes secreted were of more than one kind and could carry proteolysis beyond the amino-acid stage or, on the other hand, that cell division and consequently synthesis of amino-acid into bacterial protoplasm was proceeding at a maximum rate during this period, the apparent decrease in numbers being due to the fact that the death rate began to make itself felt at the point when the count was 2,000,000,000.

TABLE 6
B. PYOCYANEUS

Milligrams N per C C As Amino-Acid and As Coagulable Protein on Ascitic Fluid + Sugar-Free Broth			
Incubation	Amino-Acid	Coagulable Protein	Bacteria per C C
Sterile	0.441	0.846	
12 hours	0.439	0.846	88,000,000
36 hours	0.520	0.818	2,000,000,000
60 hours	0.529	560,000,000
84 hours	0.537	0.433	208,000,000
10 days	0.386	0.172	10,000,000

In order to throw more light on this apparent preference for some protein-decomposition products instead of others, it seemed useful to investigate the effects of the addition of varying amounts and kinds of amino-acids to culture mediums. Accordingly, 0.1 cc of 48-hour broth culture of *B. typhosus* was inoculated into each of five flasks of sugar-free broth which differed only in that flask 1 was ordinary sugar-free broth containing 1% peptone, flask 2 contained in addition 0.05% tyrosin, flask 3 0.2% asparagin, flask 4 0.5% glycol, and flask 5 extra peptone making a strength of 3%. The results are given in table 7 and represented graphically in chart 3 a.

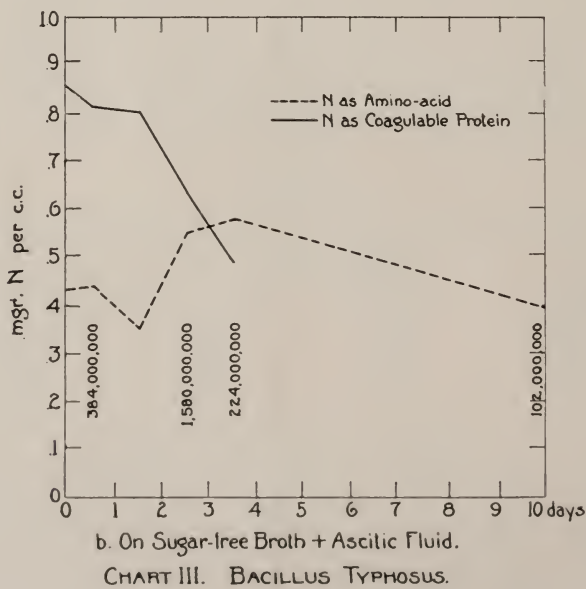
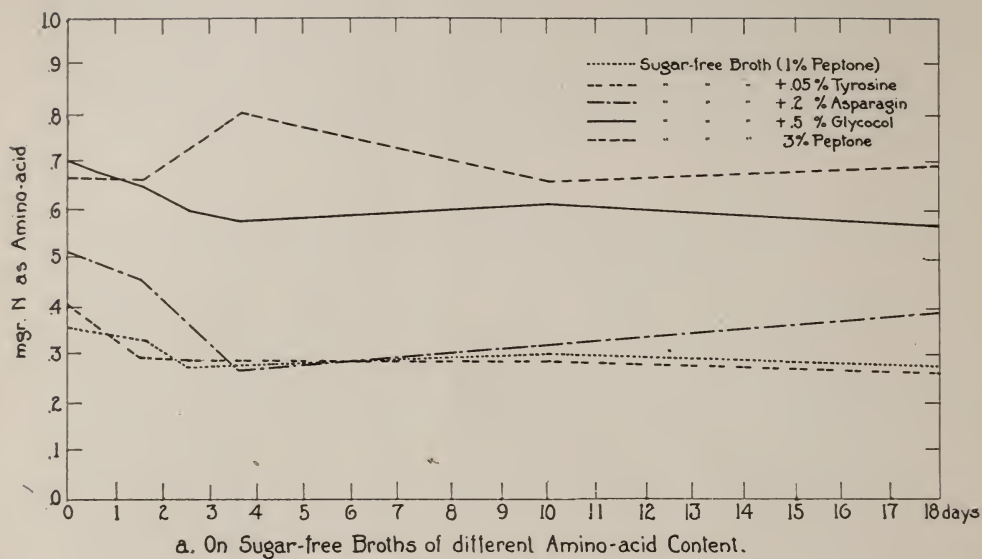


TABLE 7
B. TYPHOSUS

Milligrams N As Amino-Acid per C C on Broths of Varying Amino-Acid Content					
Incubation	1% Peptone	0.05% Tyrosin 1% Peptone	0.2% Asparagin 1% Peptone	0.5% Glycocol 1% Peptone	3% Peptone
Sterile	0.357	0.401	0.520	0.715	0.678
16 hours	0.343
18 hours	0.686
36 hours	0.321	0.295
38 hours	0.452	0.651	0.667
60 hours	0.277	0.282	0.605
90 hours	0.272	0.577	0.806
10 days	0.309	0.315	0.619	0.663
18 days	0.277	0.265	0.389	0.567	0.686

In the flask of plain sugar-free broth we note a gradual decrease in amino-acid during the first two days. This period covers the lag-phase and the period of most rapid multiplication, during which it uses those acids present in the medium which the organism is especially able to assimilate. The fact that no great change was apparent at the times when subsequent tests were made would seem to indicate that the peptone in the culture was being used, and that the resulting decomposition products were either synthesized into bacterial protoplasm or else carried beyond the amino-acid stage. However, there seems to be a certain amount of amino-acid present which remains untouched, doubtless being unsuitable as nutriment for the cells. In the tyrosin flask the same level is reached and maintained after 60 hours, indicating that the extra foodstuff in that form was readily assimilated. The flask with asparagin shows the same process in a more striking degree. It is unfortunate that no 60 hour test was made to determine whether the asparagin was used up in that length of time, or really required $3\frac{1}{2}$ days to reach the dead level as the chart seems to show. The flask which contained a considerable amount of glycocol shows a similar absolute decrease, but the level maintained is higher than that of the other flasks by just about the amount of glycocol added. (The difference in amino-acid nitrogen between the plain broth flask and the one with glycocol was 0.358 mg. per cc when sterile, and 0.354 mg. after 10 days' incubation.) This apparently indicates that glycocol is not assimilated by *B. typhosus* when other sources of nitrogen are present. In the 3% peptone flask there is very little decrease during the first $1\frac{1}{2}$ days, indicating that the free amino-acid used up is balanced by the fact that some is being set free from the peptone. After this, apparently the peptone destruction sets free more acid than is used up, but later still these are synthesized or more likely broken down into simpler substances. (Sears found that *B. typhosus* formed considerable amounts of ammonia on peptone solution.)

These results are confirmed and explained by an experiment using a medium consisting of mineral salts, glycerol and a pure amino-acid. Absolutely no visible growth of *B. typhosus* could be determined in such a medium with glycocol as a sole source of nitrogen, although it grew well on this medium when other amino-acids were introduced, and several other bacteria produced a heavy growth on the glycocol. In the typhoid-glycocol culture tubes there was no appreciable diminution of the amount of amino-acid present during 3 weeks, though the bacilli introduced remained viable throughout the period of observation.

The results obtained with the same strain on a mixture of sugar-free broth and ascitic fluid (table 8, chart 3b) are of interest in connection with the first experiment.

TABLE 8
B. TYPHOSUS

Milligrams N per C C As Amino-Acid and As Coagulable Protein in a Mixture of Broth and Ascitic Fluid			
Incubation	Amino-Acid	Coagulable Protein	Bacteria per C C
Sterile	0.430	0.859	
12 hours	0.439	0.818	384,000,000
36 hours	0.354	0.805	
60 hours	0.551	0.639	1,580,000,000
84 hours	0.581	0.488	224,000,000
10 days	0.386	Lost	102,000,000

Here the protein is attacked from the first, and there is even a slight rise in amino-acid during the first 12 hours, then a considerable fall in the next 24, followed by a rapid rise in free amino-acid, and great destruction of protein continuing even a little after the maximum count was reached. Then ensues a period during which the amino-acids are gradually used up by the germs.

The two flasks of ordinary sugar-free broth inoculated with *B. proteus* (table 9, chart 4a) indicate that, after the first drop due to the utilization of the amino-acids present, very little of the amino-acid broken from the peptone is left free in the medium, and the accumulation is not great.

TABLE 9
B. PROTEUS

Milligrams N As Amino-Acid per C C of Broth		
Incubation	Flask 1	Flask 2
Sterile.....	0.329	0.329
1 day.....	0.239	0.233
2 days.....	0.191	0.202
6 days.....	0.230	0.235
8 days.....	0.282	0.282

On undiluted ascitic fluid (table 10, chart 4c) this is even more marked. Unfortunately, determinations of coagulable protein were not made, but the idea that the small amount of free amino-acid is due to thoroughness of decomposition rather than to lack of protein is strengthened by the fact that this was the same sample of fluid on which *B. pyocyaneus* set free 0.695 mg. per c c in 12 days.

TABLE 10
B. PROTEUS

Milligrams N As Amino-Acid per C C of Pure Ascitic Fluid									
Incubation.....	Sterile	1 day	2 days	3 days	4 days	5 days	6 days	7 days	12 days
Milligrams N.....	0.077	0.060	0.054	0.043	0.026	0.031	0.054	0.032	0.026

On the mixture of broth and ascitic fluid (table 11, chart 4b) the striking destruction of coagulable protein, and the very large amount of amino-acid liberated in the first 2½ days apparently indicates that this organism attacks both protein and peptones whenever they are present, indiscriminately, and uneconomically in the case of a medium rich in both. Later much of this surplus amino-acid was used up.

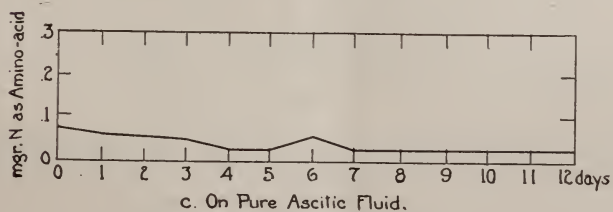
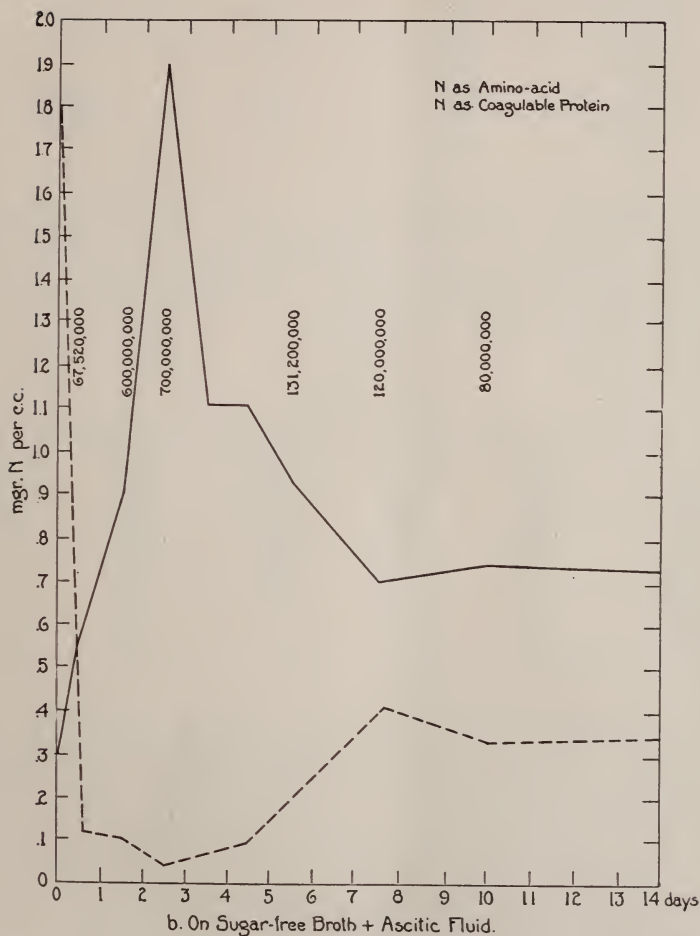
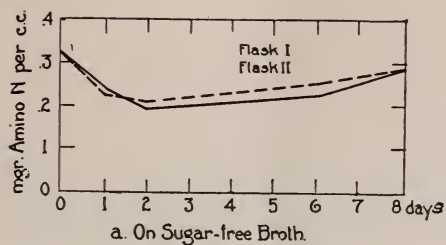


CHART IV. *BACILLUS PROTEUS*.

TABLE 11

B. PROTEUS

Incubation	Sugar-Free Broth Plus Ascitic Fluid		
	N per C C As NH ₂	N per C C Coagulable Protein	Count
Sterile	0.307	1.807	0
14 hours	0.575	0.119	67,520,000
36 hours	0.900	0.105	600,000,000
2½ days	1.980	0.049	700,000,000
3½ days	1.175		
4½ days	1.173	0.091	
5½ days	0.934	131,200,000
7½ days	0.707	0.412	120,000,000
10 days	0.743	0.337	80,000,000
2 weeks	0.732	0.348	

In this flask as in the staphylococcus flask already mentioned, it was almost impossible to obtain a clear fluid by centrifugation after the 1st week, and this probably accounts for the rise in coagulable protein noted on the 7th day.

It is interesting to compare the results on the flasks of broth reinforced with various amino-acids (table 12, chart 5) with the results on plain broth as well as with the behavior of *B. typhosus*.

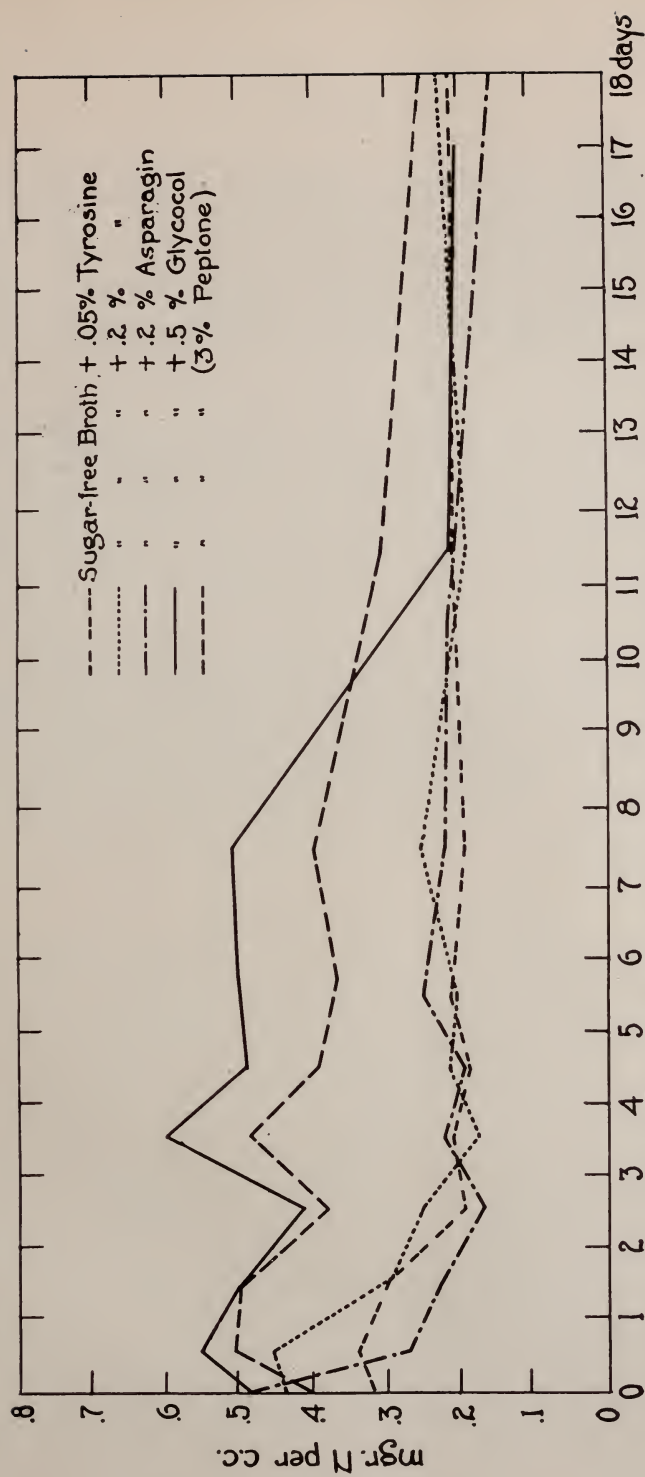
TABLE 12

B. PROTEUS

	0.05% Tyrosin	0.2% Tyrosin	0.2% Asparagin	0.5% Glycocol	3% Peptone
Sterile	0.327	0.440	0.469	0.480	0.402
12 hours	0.345	0.449	0.276	0.556	0.503
36 hours	0.309	0.292	0.220	0.496	0.499
60 hours	0.199	0.254	0.165	0.417	0.386
84 hours	0.209	0.175	0.219	0.602	0.481
4½ days	0.188	0.210	0.190	0.485	0.397
5½ days	0.212	0.201	0.257	0.491	0.372
7½ days	0.199	0.255	0.221	0.518	0.408
10½ days	0.219		
11½ days	0.209	0.188	0.208	0.297
17 days	0.204	
18 days	0.209	0.222	0.155	0.255

Here only one flask showed an initial fall in amino nitrogen content, namely, that containing asparagin. This substance is apparently assimilated with considerable vigor at the same time that the peptone is being broken down, and the "dead level" of useless amino-acid is reached in 2½ days. In the case of tyrosin the amounts set free are in excess of the amounts liberated during the first twelve hours but in a few days the content is about the same as that of the asparagin flask. The glycocol flask shows a very different curve from that produced with *B. typhosus*. Here we have an initial rise due to the attack on peptone, followed by fluctuations and a 3-day period of no great change, and then a rapid fall between the 7th and 11th days, when the "dead level" was reached. In the 3% peptone flask the initial increase is more marked than in any of the others, which is what might be expected, considering the readiness of the organism to attack such substances, and the fact that there is three times as much peptone present as in each of the other four flasks.

The results on the synthetic medium (mineral salts, glycerol and a single amino-acid) agree with these findings. While *B. proteus* did not use up



On Sugar-free Broths of different Amino-acid Content.

CHART V. *BACILLUS PROTEUS*.

glycocol as rapidly as some other bacteria did, it grew fairly well, proving that it can derive nitrogen from this acid if "starved to it." These peculiarities seem worthy of further study. It was thought possible that this difference might have some bearing on the liquefaction of gelatin, which yields a large percentage of glycocol on hydrolysis. However, several bacteria (*B. paratyphosus*, *B. coli*, *B. fecalis-alkaligenes*) incapable of liquefying ordinary gelatin mediums, thrive on these glycocol solutions. Investigations using a variety of amino-acids for a considerable number of organisms promise interesting results, and are well under way.

SUMMARY

Four organisms were studied, two of which are strongly proteolytic on ordinary mediums (*B. proteus* and *B. pyocyaneus*), one moderately so (*staphylococcus*), and one (*B. typhosus*) which does not show the marked activity of the others. On mediums containing ascitic fluid all four destroyed considerable amounts of coagulable protein during the first 36 hours. *B. pyocyaneus* was the only one which did not do so in the first 12. In the case of the *staphylococcus* this digestion proceeded at a fairly uniform rate; *B. typhosus* and *B. pyocyaneus* apparently did not attack the coagulable protein vigorously until the more easily assimilable substances had been used; *B. proteus* seems to attack all the nitrogenous materials at once without discrimination. *B. proteus* and *B. typhosus* could both assimilate tyrosin and asparagin; *B. proteus* could make use of glycocol; *B. typhosus* could not.

DISCUSSION AND CONCLUSIONS

Evidently, the ability of a given cell to assimilate amino-acids does not result directly from their simplicity of structure and solubility in water; otherwise glycocol, which is the simplest and is readily soluble, would be the most easily used. We have been led to believe that the intracellular proteins synthesized by bacteria are specific, in view of various phenomena connected with immunity. It seems reasonable to suppose that this individuality may rest to some extent on the ability to absorb certain substances characteristic of those proteins. Sperry and Rettger found that pure crystallized proteins cannot be attacked by bacteria in the absence of decomposition products; experiments made in this department by Diehl seem to indicate that bacteria will not elaborate a ferment against a certain protein if grown on a medium containing none of the amino-acids found in that protein. The idea of individual requirements in amino-acid by various kinds of bacteria lends color and significance to these observa-

tions. We can think of the free amino-acids present in a culture containing a protein and its split products as both "building stones" in the protein molecule, and as possible "stepping stones" for the bacteria. Then the avidity with which an organism attacks a protein would be in direct proportion to the amount and variety of free amino-acids present which are represented in the structure of the protein molecule and which that particular kind of cell can assimilate. If only a few acids fulfilling both these requirements are found in the medium, the stimulus to proteolysis would not be great, at least until possibly a greater quantity and variety was set free by the first feeble attack. An organism exhibiting what might be called a catholicity of taste in amino-acids would have an increased number of approaches to the protein molecule. Further investigations will show whether or not one is justified in stating that *B. proteus*, for instance, is such an organism.

In the meanwhile we are certainly justified in concluding that the nature of the particular protein decomposition products present play a very important part in metabolism, as the power to assimilate a given amino-acid is not necessarily common to all bacteria, but is due to factors which may be absent in some varieties.

THE EFFECT OF THORIUM X ON ACTIVE ANAPHYLAXIS IN THE GUINEA-PIG

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It has been suggested that the greater part of the difference between tuberculosis in children and in adults is explicable on the basis of an allergic condition in the adult. Early in his work Koch recognized that an animal injected with tubercle bacilli reacted distinctly differently locally after the second injection than after the first.¹ It also seems well established that the chronic cavity and fibroid type of tuberculosis is the reaction of a resistant organism while the rapidly progressive military type is the result of invasion in a highly susceptible organism. It was with a view to obtain information in regard to the significance of the allergic condition with respect to the local reaction to tubercle bacilli and to the systemic susceptibility that the following experiments were made.

It was thought that if it was possible by some means to reduce the allergic condition in guinea-pigs such animals would reveal marked differences in their reactions as compared with allergic animals not thus treated.

The results of the work by v. Heinrich² and by Schiff³ indicated that the proposed plan for determining the importance of allergy in tuberculosis would be feasible. By means of the roentgen ray v. Heinrich was able markedly to influence the formation of "sensibilisin." Using diphtheria antitoxin (horse serum) as the protein solution, and giving 0.01 c c for sensitizing and from 0.05-0.5 c c as intoxicating doses, he found that one erythema dose (3 Kaloms) of roentgen ray given any time within 14 days after the sensitizing dose, the intoxicating dose given three weeks later, would so influence the lymphoid tissues that, as he believed, antibody formation was interfered with and thus also the formation of the anaphylactic poison. The greatest effect was noted coincidentally with the maximum effect on the lymphoid tissues. Roentgenization immediately before the intoxicat-

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¹ Corper, H. J.: *Am. Rev. Tuberculosis*, 1917, 1, p. 407.

² *Centralbl. f. Bakteriol.*, I, O., 1913, 70, p. 421.

ing dose was without effect. In experiments on passive anaphylaxis, the roentgenized guinea-pig serum conferred no sensitiveness on the recipient, in contradistinction to the serum of untreated animals, thus demonstrating further the absence of antibody formation. He suggested that the experiments would be made better with thorium X, but was unable to obtain any. Peptone shock—differing from anaphylactic shock—was not influenced by roentgenization. He believes with Seligmann⁴ that inability to passively transfer anaphylaxis from tuberculous guinea-pigs is due to the large amount of destruction of lymphoid tissue suffered by such animals. Schiff,³ realizing that benzene, on account of its destructive action, was not as suitable as the roentgen ray, tried its effect on anaphylaxis produced by sheep serum, 0.02 c c subcutaneously for sensitizing and from 0.001-0.02 for intoxicating on the 18th day. The benzol was given in doses of 0.01 c c intraperitoneally, 7 injections at 4-day intervals, and of 0.03 c c intraperitoneally, 8 injections at 2-day intervals. The fatal doses of intoxicating protein was the same in the treated and untreated guinea-pigs. Differences were noted only on the border line, 57% of the small dose benzene animals and 17% of the controls dying, while 17% of the large dose benzene animals and 71% of the controls died. He believed the increased or diminished susceptibility to be due to the influence of the benzene on the tissues that form antibodies; that is, the hematopoietic system, and concludes that benzene influences the anaphylactic reaction in actively prepared animals, small doses of benzene increasing the susceptibility and large doses diminishing it.

On account of the availability, regulation of dosage, and administration, thorium X was used in the experiments now reported. Thorium X is easily administered in 0.9% sodium chlorid solution, is not influenced by boiling and its strength is easily and quickly determined by means of a simple electroscope.⁵ The radio-thorium from which the thorium X was prepared for use was contributed by Dr. Herbert N. McCoy, Carnotite Reduction Co., Chicago.

For comparison, three separate protein mixtures, egg white, normal horse serum and milk were used for producing anaphylaxis. The thorium X was given at different intervals before and after sensitiza-

³ Zeitschr. f. Immunitätsf., 1914-15, 23, p. 61.

⁴ Zeitschr. f. Immunitätsf., 1912, 14, p. 419.

⁵ Corper, H. J.: Am. Rev. Tuberculosis, 1918, 2, p. 587.

TABLE 1
THE EFFECT OF THORIUM X ON ANAPHYLAXIS BY EGG WHITE

Experiments	Thorium X		Maximum Reaction	Time of Maximum Reaction	Lowest Leukocyte Count	Time in Days of Lowest Leukocyte Count		Leukocyte Count on Day of Second Protein Injection
	Dose in Units	Administration				After Sensitization	After Injection Thorium X	
Series I Expers. 1, 2, 3	0		Died	20"	7,750			8,250
			Died	20"	13,000			15,250
			Died	25"	8,250			8,500
Single large injection of Thorium X with sensitizing injection. Expers. 4, 5	35	With 1st (sensitizing) injection of protein	Died	30"	1,500	14	14	2,500
			Died	25"	1,250	13	13	2,100
Leukocytes maintained low throughout entire incubation period. Expers. 6, 7, 8, 9	3 to 5	Injections begun 4 days before 1st injection of protein and repeated daily or every second day as necessary	Died	27"	2,150	17		2,250
			Died	42"	1,500	18		1,750
			Died	50"	1,625	17		2,000
			Died	36"	1,250	19		1,250
Small repeated injections of Thorium X. Expers. 10, 11, 12	0.1	Injections begun 3 days before first protein injection and repeated every third day throughout	Died	20"	7,500	10		8,750
			Died	40"	5,250	17		7,000
			Neg.		7,750	12		8,500
Single large injection of Thorium X 7 days before second protein injection. Expers. 13, 14, 15	35	Single injection 7 days before second protein injection	Died	35"	2,500	19	7	2,500
			Died	25"	1,250	18	6	1,500
			Died	30"	2,000	17	5	2,100
Series II Expers. 16, 17, 18	0		Died	15"	10,000			10,500
			Died	20"	8,750			9,500
			Died	20"	8,000			8,000
First injection protein given when leukocytes were low. Expers. 19, 20	40	Single large injection 6 days before first protein injection	Died	20"	1,250	4	10	5,000
			Died	20"	1,000	5	11	5,000
Single large injection of Thorium X given with second protein injection. Expers. 21, 22, 23	40	Single large injection with second protein injection	Died	20"	8,000			8,750
			Died	15"	8,750			10,000
			Died	25"	7,250			8,750

Series I includes animals 1-15 in which the interval between the first and second injection of egg white was 19 days. Series II includes animals 16-23 given the same dose of egg white (0.1 cc with 0.1 cc of distilled water intraperitoneally for the first (sensitizing) injection and 1 cc plus 1 cc of distilled water intraperitoneally for the second (intoxicating) injection), but the interval between the first and second injection of egg white being 17 days.

A unit is an arbitrary index of the amount of Thorium X that will completely discharge an electroscope of certain type in one minute.⁵ Expressed in lethal doses 100 units are fatal to a guinea-pig about 400-600 gm. in weight in 15 days while 50 units are not lethal, 250 units kill in 10 days, 500 in 7 days and 1,000 in 6 days.

tion, and before and with the second (intoxicating) dose of protein, and in different amounts. Daily leukocyte counts were made to note the effect of the thorium X and the relation between the number of circulating leukocytes and anaphylaxis. The experiments with egg white, prepared by mixing fresh egg white with an equal volume of sterile distilled water and filtering, all resulted in fatal anaphylaxis so that only the inhibitory effect of the thorium X, if such occurred, could be noted. The results are given in table 1.

It is to be noted that thorium X in large doses, given before (6 days) or coincident with the sensitizing injection of egg white, or before (7 days) or with the second injection, had no appreciable effect on the fatal anaphylaxis from the second injection of egg white. Likewise, repeated injection of thorium X sufficient to maintain a leukopenia as low as 2,000 leukocytes per c mm of peripheral blood throughout the incubation period, or very small repeated injections not appreciably affecting the peripheral leukocytes, had just as little effect on the anaphylactic shock from the second injection of egg white.

The second set of experiments in which normal horse serum was used resulted in less severe anaphylactic symptoms on the second injection of the serum in the controls and therefore was more suitable for determining slight effects on anaphylaxis by the thorium X. Examination of table 2, however, reveals no consistent effect of the thorium X on the severity of the anaphylaxis by the normal horse serum. It seems that, if anything, the thorium X treated animals were more severely affected by the second protein injection than the controls.

In the experiment with milk protein the results of the second injection were not fatal, and thus permitted notation of slight variation in the anaphylactic reaction as affected by the thorium X (table 3).

As a whole the experiments with milk protein are corroborative of those with normal horse serum in that thorium X in large doses given before (7 days) or coincident with the first injection of milk or before 8 days, or with (5 hours before) the second injection had no appreciable effect on the severity of the anaphylaxis. Likewise repeated injection of thorium X, sufficient to maintain a leukopenia as low as 2,000 per c mm of peripheral blood throughout the interval of 16 days, between the first and second injection of milk, or very small repeated injections not appreciably affecting the peripheral leukocytes had just as little effect on the severity of the anaphylactic symptoms.

TABLE 2
THE EFFECT OF THORIUM X ON ANAPHYLAXIS BY NORMAL HORSE SERUM

Experiments	Thorium X		Maxi- mum Reac- tion	Time of Maxi- mum Reac- tion	Lowest Leuko- cyte Count	Time in Days of Lowest Leukocyte Count		Leuko- cyte Count on Day of Second Protein Injection
	Dose in Units	Administration				After Sensi- tiza- tion	After Injec- tion Thor- ium X	
Series I Expers. 1, 2, 3	0		+	1'	6,000			7,750
			+	1'	9,500			9,500
			+++	1'	6,250			7,250
Single large injection of Thorium X with sensitizing injection. Expers. 4, 5, 6	35	With first injection of protein	Died	45"	1,500	9	9	4,250
			Died	25"	1,125	9	9	2,750
			++	1'	2,350	8	8	4,000
Leukocytes maintained low throughout entire incubation period. Expers. 7, 8, 9	3 to 5	Injections begun 4 days before first injection of protein and repeated daily or every second day as necessary	+++	1' (dead 26')	1,500	9		1,600
			Died	2'	1,250	15		1,300
			+++	1'	1,250	15		1,600
Small repeated injections of Thorium X. Expers. 10, 11, 12	0.1	Injections begun 3 days before first protein injection and repeated every third day throughout	Died	45"	5,250	13		6,000
			++	30"	8,000	11		8,500
			++	1'	6,250	10		8,000
Single large injection of Thorium X 7 days before second protein injection. Expers. 13, 14, 15	35	Single injection 7 days before second protein injection	++	40" (died 18')	1,000	19	7	1,000
			+++	30" (died 36')	750	19	7	750
			Died	1¼'	750	19	7	750
Series II Expers. 16, 17, 18	0		+++	45"	8,000			8,750
			+++	45"	7,500			8,750
			+++	45"	7,000			7,750
The first injection of protein given when leukocytes were low. Expers. 19, 20, 21		Single large injection 6 days before first protein injection	+++	1'	1,000	2	8	5,000
			+++	1¼'	1,250	3	9	4,750
			+++	45"	1,000	2	8	5,500
A single large injection of Thorium X given with second injection of protein. Expers. 22, 23, 24	40	Single large injection with second protein injection	++	30"	7,250			8,000
			Died	50"	6,250			7,500
			Died	1½'	7,500			10,000

Series I includes animals 1-15 in which the interval between the first and second injection of normal horse serum was 19 days. Series II includes animals 16-24 given the same dose of normal horse serum [0.1 cc with 0.1 cc of distilled water intraperitoneally for the first (sensitizing) injection and 1 cc with 1 cc of distilled water intraperitoneally for the second (intoxiating) injection] but the interval between the first and second injection of horse serum being 17 days.

The reactions are graded into mild +, moderate ++, and severe +++ with subsequent recovery of the animal.

TABLE 3
THE EFFECT OF THORIUM X ON ANAPHYLAXIS BY MILK PROTEINS

Experiments	Thorium X		Maxi- mum Reac- tion	Time of Maxi- mum Reac- tion	Lowest Leuko- cyte Count	Time in Days of Lowest Leukocyte Count		Leuko- cyte Count on Day of Second Protein Injec- tion
	Dose in Units	Administration				After Sensi- tiza- tion	After Injec- tion Thor- ium X	
Controls. Expers. 1 and 2	0		+++	45"				
			+++	1'				
First injection of protein given when leuko- cytes were low. Expers. 3, 4	40	Single large injection 7 days before the first pro- tein injection	++	45"	1,750	0	7	6,250
			+++	1'	1,000	4	11	4,500
Single large injection of Thorium X with sensitizing injection. Expers. 5 and 6	40	With first injection of protein	+++	1'	1,250	8	8	3,750
			++	30"	1,250	12	12	4,500
Leukocytes maintained low throughout entire incubation period. Expers. 7, 8	5-10	Injections begun 7 days before first protein in- jection and repeated as necessary	++	45"	2,500	0		2,750
			+++	45"	1,250	6 and 12		2,250
Small repeated injections of Thorium X. Expers. 9, 10, 11	0.1	Injections begun 2 days before the first protein injection and repeated every third day throughout	+++	45"	6,250	10		7,000
			+++	45"	7,750	12		8,000
			+++	30"	7,250	9		7,500
Single large injection of Thorium X before the second protein injection. Exper. 12, 13, 14	40	Single injection 8 days before the second protein injection	+++	1'	1,250	15	7	1,500
			++	30"	2,300	16	8	2,400
			+++	30"	2,500	16	8	2,500
A single large injection of Thorium X given with the second injection of protein. Expers. 15, 16	40	Single large injection 5 hours before second protein injection	+++	1'	5,000			6,500
			+++	1½'	6,750			8,000

The guinea-pigs in this experiment all received 0.5 cc of milk for the first injection intraperitoneally and 5 cc of milk for the second injection intraperitoneally, the interval between the injections being 16 days.

In order to note whether there was any direct relation between the size of the dose of thorium X or the leukocyte level in the peripheral blood and the severity of the symptoms, a series of guinea-pigs were given different amounts (from 1-500 units) of thorium X seven days before the second protein injection. Normal horse serum was used as the protein mixture because the results obtained with it allowed of notation of variations of either an inhibitory or enhancing influence

(table 4). In interpreting these results, it is to be borne in mind that 100 units of thorium X are lethal to a guinea-pig in 15 days, 250 units in 10 days, 500 units in 7 days, and 1,000 units in 6 days; thorium X acting as a chronic poison; 50 units are nonlethal.

TABLE 4
EFFECT OF DIFFERENT AMOUNTS OF THORIUM X AND OF DIFFERENT LEUKOCYTE LEVELS ON ANAPHYLAXIS BY NORMAL HORSE SERUM

	Thorium X Dose in Units	Maximum Reaction	Time of Maxi- mum Reaction	Lowest Leukocyte Count	Time in Days of Lowest Leukocyte Count		Leukocyte Count on Day of Second Protein Injection
					After Sensi- tiza- tion	After Injection of Thor- ium X	
1 2 3	0	+++ +++ +++	1' 45" 45"				
4 5	1	Died Died	1'10" 1'25"	5,000 4,500	16 16	5 5	5,500 5,000
6 7	2	Died ++	2' 15"	4,000 4,500	15 16	4 5	4,750 5,000
8 9	5	+++ +++	45" 45"	2,500 1,500	18 16	7 5	2,500 2,500
10 11	10	+++ ++	45" 45"	3,250 2,500	18 16	7 5	3,250 2,500
12 13	20	+++ ++	1' 45"	1,250 1,750	17 18	6 7	1,500 1,750
14 15	50	+++ ++	45" 45"	2,500 1,750	16 17	5 6	3,000 1,750
16 17	100	Died +++ (Died 24')	2¼' 2'	1,000 750	17 16	6 5	1,500 1,000
18 19		++ (Died 2 days) +++ (Died 5')	1' 2'	500 500	18 18	7 7	500 500
20 21	500	++ (Died 5') +++ (Died 18')	2' 2'	500 500	18 17	7 6	500 500

The thorium X was injected intraperitoneally 7 days before the second protein injection was given, and the interval between the first and second protein injection was 18 days.

With the exception of the animals given one unit, in which case the results are unexplained—Schiff found small doses of benzene to increase the severity of the reaction—and those given lethal doses of thorium X, the results are fairly uniform in that they indicate that thorium X *per se* or the number of leukocytes found in the peripheral blood have no influence on the symptoms produced in guinea-pigs by the second injection of horse serum. When a lethal dose of thorium X had been given the second injection of horse serum simply hastened death probably because of the combined action of two poisons—thorium X and anaphylatoxin.

DISCUSSION

The object of these experiments was to obtain a method applicable to the study of one of the problems in tuberculosis — that of allergy. In this the method planned proved impractical. Although the experiments were not planned to give slight differences, they seem to have been of sufficient accuracy to indicate either that the roentgen ray and benzene act differently from thorium X or that the observations with the roentgen ray and benzene are incorrect and require corroboration. The work should be repeated, using the roentgen ray, benzene and thorium X at the same time, and with a large series of animals to rule out accidental differences.

SUMMARY

Thorium X given in about $\frac{1}{2}$ the lethal amount seven days before or coincident with the primary injection of egg white, normal horse serum or milk proteins, or 7 days before or with the second injection of these proteins had no appreciable effect on the severity of the anaphylactic symptoms in guinea-pigs on reinjection of protein 16 to 18 days after the primary injection. Likewise, the repeated administration of smaller doses of thorium X sufficient to maintain a leukopenia as low as 2,000 leukocytes per cmm throughout the entire interval of 16 to 18 days between the first and second injections, or very small repeated injections not appreciably affecting the number of peripheral leukocytes, had just as little effect on the severity of the anaphylactic symptoms.

There was no direct relation noted between the anaphylactic symptoms and the leukocyte counts as affected by thorium X. In the larger doses the effect observed seemed to be due to the combined action of two toxic substances — the thorium X, a chronic poison, and the anaphylatoxin, an acute poison.

ANTIBODIES IN THE CHICK

HAL W. SHERMAN

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Klemperer¹ found that diphtheria antitoxin was transmitted from immunized fowls to the vitellus of the egg. Dzierzowski² obtained the same results, and in addition reported that the blood of chicks hatched from such eggs contained antitoxin. Figari³ fed tubercle bacilli to hens and demonstrated specific agglutinins in the eggs; he found no agglutinins in control eggs.

The scope of my work was to study natural antibodies of hens, chicks, and chick embryos. Lysins for sheep, goat, rabbit and human corpuscles have been estimated; also complement. Blood was obtained from the wing vein of the hen, and the jugular veins of chicks. To get blood from the embryo without contamination by other fluids is more difficult. The incubated eggs were placed on a candling device and the shell and outer membrane over the air chamber removed. As the inner membrane came into view xylol was brushed carefully over it; the membrane then became transparent, the blood vessels stood out prominently, and one of the largest vessels could now be punctured with a needle and the embryo bled to death into the air chamber, from which the blood was removed with a pipet before clotting occurs. Great care was exercised not to go through the membrane with the needle, as then other fluids came out with the blood and air gained entrance into the egg substance with consequent bulging of the inner membrane and shell. Anywhere from $\frac{1}{2}$ -1 c c of blood could be obtained from an embryo in this way.

Several different batches of chick serums were tested at different times and for convenience the results are given in table 1. In no case was lysin found in any embryo except those of 21 days' incubation, and in this case the chicks were pecking their way out of the shell. Their serum contained lysin for rabbit erythrocytes only, 0.1 c c of serum being required to lake completely 0.1 c c of a 1% erythrocyte suspension. Complement was found in the embryo serums of 17 and

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¹ Arch. f. exper. Path. u. Pharmacol., 1893, 31, p. 356.

² Centralbl. f. allg. Path. u. path. Anat., 1901, 12, p. 715.

³ Centralbl. f. Bakt., I, O., 1907, 39, p. 75.

21 days' incubation; it required 0.05 c c of the serum of the younger embryo and 0.1 c c of that of the older to lake completely 0.1 c c of a 1% suspension of rabbit erythrocytes when 1 unit of amboceptor (dog) was added. No embryo serum examined contained complement for sheep, goat or human erythrocytes. The embryo serums were not anticomplementary. Lysins and complement were not present in the amniotic or allantoic fluids or in fresh nonincubated eggs.

TABLE 1
RESULTS OF DIFFERENT TESTS

Age of Chick	Complement Titers Action of Serum + 1 Unit of Amboceptor on Erythrocytes of				Lysin Titers Action of Serum + 1 Unit of Complement (Guinea-pig Serum) on Erythrocytes of			
	Sheep (Rabbit Ambo- ceptor)	Goat (Rabbit Ambo- ceptor)	Human (Rabbit Ambo- ceptor)	Rabbit (Dog Ambo- ceptor)	Sheep	Goat	Human	Rabbit
Pecking at shell	no lysis	no lysis	no lysis	+++ (0.1)	no lysis	no lysis	no lysis	+++ (0.1)
1 hour	no lysis	no lysis		+++ (0.01)	no lysis	no lysis		+++
10 hours	no lysis	no lysis		+++	no lysis	no lysis		+++
24 hours	no lysis	no lysis		+++	no lysis	no lysis		+++
24 hours	no lysis	no lysis	no lysis	+++ (0.1)	+++ (0.2)	no lysis (0.3)	no lysis (0.3)	+++ (0.1)
24 hours	no lysis	no lysis		+++ (0.01)	+++ (0.05)	no lysis		+++
48 hours	+++	+++	+++	+++	+	+++ (0.1)	+++ (0.1)	+++
72 hours	+++	+++		+++ (0.01)	+++	no lysis		+++ (0.01)
21 days	+++ (0.1)	+++ (0.1)	+++ (0.1)	+++ (0.1)	+++ (0.1)	no lysis	+++ (0.1)	+++
Full grown	+++ (0.1)	+++	+++ (0.1)	+++	+++	+++	+++	+++

0.1 c c of a % erythrocyte suspension was used for the older chicken while in the newly hatched 0.1 c c of a 1% suspension was taken.

Unless otherwise stated by figures in parentheses, the amount of chick serum used was either 0.05 or 0.03 c c.

+++ means complete laking; ++ partial laking; + slight laking.

In a previous paper⁴ I reported that lysins and complement are inappreciable in the youngest swine embryos; they were found in varying amounts, however, after the 9th week of gestation. Whether these antibodies were autochthonous or transmitted through the placenta from the maternal blood seems impossible to determine, although their detection only in the 9th week would seem to make more probable the view that they came through the placenta. Histologists have shown a less intimate relationship between the fetal and maternal blood vessels in early embryonic life than later. If this anatomic

⁴ Jour. Infect. Dis., 1919, 24, p. 1.

difference permits of easier transmission of nutrition to the fetus, then why not also of antibodies? The study of the chick embryo was undertaken to avoid the complication of the transmission of antibodies from the maternal blood through the placenta. No antibodies were found in the chick embryos before the 21st day of incubation when all the embryos examined were pecking at the shell. This is an entirely different condition than that presented by the swine fetus. How are we to account for the lysins of the 21-day embryo and the lack of them in the 19-day embryo? There are two physiologic differences in the embryos of these ages. In the 19-day embryo respiration is maintained by the capillaries of the internal membrane exclusively, and in the 21-day embryo this capillary network is aided by occasional inspiration by the lungs; this is of doubtful significance. It is necessary to consider another factor. With the chick pecking on the shell, it seems possible that there may be a stimulation of secretion from gastro-intestinal glands with subsequent absorption of antibodies into the blood. Whether antibodies and complement occur in the gastro-intestinal secretions is a moot subject. R. Neumann⁵ has described a dog in which the large intestine was cut off from the small intestine and the ends tied; the free end of the small intestine was sutured to an artificial anus; after several months the secretion from the large intestine was collected and found to contain hemolysins qualitatively the same as those of the blood. Whether lysins in the intestines were derived from the blood is a question; there may be no relationship, but this seems unlikely. Metchnikoff⁶ believed that the fixatives of the blood serum were set free as leukocytes disintegrate. Is there an increased leukocytolysis during the 20th or 21st day of incubation that can account for the presence of lysins in the 21-day and not in the 19-day embryos? There was no increase in the lysin titers of the swine embryos⁴ in the last days of intra-uterine development. The source of the lysin found in the hatching and newly hatched chick remains obscure.

⁵ Arb. a. d. path. anat. Inst. zu Tübingen, 1911, 7, p. 546.

⁶ Immunity in Infective Diseases, 1905, p. 98.

HEMOLYTIC STREPTOCOCCI IN THE THROAT OF THE DOG

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In view of the interest in hemolytic streptococci it seemed desirable to study the prevalence and characteristics of such streptococci in the throat of the dog.

Cultures were made by swabbing the throats of live dogs, endeavoring particularly to reach the tonsils and nasopharynx, and inoculating aerobic 10% goat blood-agar plates. Hemolytic colonies were transferred to plain broth, from the broth cultures blood agar plates made, and the single hemolytic colonies studied further. The hemolytic colonies on the plates were all of the beta type of hemolysis; that is, the hemolytic zone surrounding the colony was from 2-4 mm. in diameter and the margin was sharp and distinct. Only those cocci were chosen for further study that grew into long chains in broth. A total of 21 strains was thus secured. Stock cultures were made on plain blood-agar slants. Typical hemolytic streptococci were isolated from the throat in 39% of 32 dogs examined.

The chains in broth cultures varied from 15-100 cocci. The individual cocci were spherical or slightly flattened at the adjoining poles. In most of the smears, some chains contained large, irregular, swollen cocci. No capsules were observed.

Sixteen of the 21 strains were pathogenic for mice when 1 c c of an 18-hour broth culture was inoculated intraperitoneally; the mice dying within 24 hours.

All the 21 strains brought about complete laking when 0.5 c c of broth culture was transferred to 0.5 c c of a 5% suspension of washed rabbit erythrocytes and the mixtures incubated for 2 hours at 37 C. in a water-bath.

For bile solubility, 0.2 c c of ox bile were added to 1 c c of broth culture and observed after one hour incubation at 37 C. None of the 21 strains were soluble in bile by macroscopic and microscopic observation.

The growth of the streptococci was scanty in plain broth; there was a thin, granular, somewhat adherent sediment that in the majority

of cases extended up one side of the tube. The supernatant fluid was clear.

The fermentative reactions were determined in Hiss sheep serum medium, containing, respectively, 1% of dextrose, inulin, lactose, maltose, mannite, raffinose, saccharose, salicin and starch; also the plain medium without added carbohydrate. The results in the sugar mediums are given in table 1. There were no changes evident in the inulin and plain mediums. There was no gas produced in any sugar.

TABLE 1
FERMENTATIVES REACTIONS IN SUGAR MEDIUMS

No. of Strain	Litmus Milk	Carbohydrates							
		Dex-trose	Lac-tose	Mal-tose	Man-nite	Raffi-nose	Saccha-rose	Salicin	Starch
1	A C	A C	A C	A C	—	—	A C	A C	—
2	A C	A C	A C	A C	—	—	A C	A C	—
3	A	A C	A	A C	—	—	A C	A C	—
4	A	A C	A	A C	—	—	A C	A C	—
5	A C	A C	A C	A C	—	A	A C	A C	—
6	A C	A C	A C	A C	—	—	A	A C	A C
7	A C	A C	A C	A C	—	—	A C	A C	A C
8	A C	A C	A C	A C	—	—	A C	A C	A C
9	A	A C	A C	A C	—	—	A C	A C	A
10	A C	A C	A C	A C	—	—	A C	A C	A C
11	A C	A C	A C	A C	—	—	A C	A C	A
12	A C	A C	A C	A C	—	—	A C	A C	A
13	A C	A C	A C	A C	—	—	A C	A C	A
14	A C	A C	A C	A C	—	—	A C	A C	A
15	A C	A C	A C	A C	—	—	A C	A C	A
16	A C	A C	A C	A C	—	—	A C	A C	A
17	A C	A C	A C	A C	—	—	A C	A C	A
18	A C	A	A C	A C	—	—	A C	A C	A
19	A C	A	A C	A C	A	—	A C	A	A
20	A C	A C	A C	A C	A	—	A	A C	A
21	A	A C	A	A C	—	—	A C	—	A

A = acid to litmus.

C = coagulation within 7 days.

In neutral red and methylene blue milk no decolorization was observed. Coagulation was produced by all strains except strains 3, 4, 9 and 21.

According to Holman's¹ classification of hemolytic streptococci, 18 strains correspond to *Streptococcus pyogenes* (ferment lactose and salicin but not mannite); two strains to *Streptococcus infrequens* (ferment lactose, salicin and mannite), and one strain to *Streptococcus anginosus* (ferment lactose but not mannite or salicin). Broadhurst² found no *anginosus* but 4 strains of *Streptococcus infrequens* and 7 strains of *Streptococcus pyogenes* in the throat of 12 dogs.

¹ Jour. Med. Research, 1918, 34, p. 377.

² Jour. Infect. Dis., 1915, 17, p. 277.

My results indicate that *Streptococcus pyogenes* can be roughly divided into two fairly even groups depending on the reaction in the starch medium (see table); whether this is of any significance remains undetermined.

Only three of the *pyogenes* strains were nonpathogenic for mice, and two of these were starch fermenters. The only raffinose fermenter was pathogenic. Both of the strains of *Streptococcus infrequens* were nonpathogenic. The one *Streptococcus anginosus* was pathogenic.

A serum for the study of the antibodies of the hemolytic streptococci was obtained by the intraperitoneal inoculation of chickens at 5-day intervals with the *pyogenes* strain. One chicken was immunized over a period of four months increasing the amount injected so that at the last inoculation it received the organisms from 3 liters of ascitic broth. The agglutinins reached their highest titer during the 3rd month of immunization. At that time a dilution of 1:640 agglutinated the homologous strain; afterward the titer dropped so that only a dilution of 1:160 agglutinated. This same serum agglutinated the *infrequens* streptococcus in a dilution of 1:40 at the 4th month, which was the highest titer it ever reached. The dog *anginosus* streptococcus as well as human strains of *pyogenes*, *infrequens* and *anginosus* never agglutinated in higher dilution than 1:10.

The immune chicken serum fixed complement completely with *anginosus* as antigen, not at all with *infrequens*, and only partially with the homologous *pyogenes* strain. There was no fixation with the human strains.

The opsonins were increased definitely above normal for the homologous strain (index 4.3), for a human *pyogenes* strain (index 3.5), but not for *anginosus* and *infrequens* strains.

SUMMARY

Most of the hemolytic streptococci in the throat of the dogs studied were of the *pyogenes* type. No close relationship could be demonstrated between the groupings of the dog streptococci according to the results of fermentation tests and the results of tests with antistreptococcus chicken serum.

A RAPID HYDROGEN ELECTRODE METHOD FOR DETERMINATION OF HYDROGEN ION CONCENTRATIONS IN BACTERIAL CULTURES OR IN OTHER TURBID OR COLORED SOLUTIONS

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Recently the determination of hydrogen ion concentrations has come into such general use in biologic work that the question often arises as to the choice of a method for this purpose.

The colorimetric method has proven suitable for most routine bacteriologic work, and has the advantage of yielding a large number of determinations in a given period of time. Its limitations however are noteworthy. In general, it does not permit of more than a fair degree of accuracy especially for colored or turbid solutions; for opaque solutions it cannot be used; when large percentages of salt or protein are in solution, its results are not dependable because of the so-called "salt" and "protein errors" of indicators; and the preparation of the standard solutions, in which one is required to assemble a great number of calibrated burets, pipets, bottles, etc., and to recrystallize, dry to constant weight and accurately weigh a number of reagents, many of which are difficult to obtain, makes it a cumbersome method.

On the other hand, the extreme accuracy possible by the hydrogen electrode method, with potentiometer and constant temperature device, is seldom required in biologic investigation. Moreover, the cost of such equipment is prohibitive for many laboratories.

More simplified outfits are described in texts on physical chemistry, but the obvious objection to them is the relatively enormous amount of time consumed in saturating the solution with hydrogen gas. This difficulty is obviated by observing the principle suggested by Hasselbach¹ in which the solution and gas are brought into equilibrium in a few minutes by shaking in a closed vessel.

The vessel here described possesses no advantages over those described by Clark² and by McClendon and others except that it is easily constructed, even by one of only average technical skill, and from such material as is

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¹ Biochem. Ztschr., 1911, 30, p. 317.

commonly found in any biologic laboratory. In urging the adoption of this method, simplicity of apparatus and technic should be its recommendations; but it seems that the method has been too much avoided by biologists, who, though recognizing its value, have regarded it as one belonging exclusively to the physical chemist. Nothing is perhaps more to blame for this, however, than the multiplicity of symbols, formulas, measurements, etc., which only confuse rather than enlighten the uninitiated. An attempt at simplifying the relations of the measurements to the mathematics of the method is therefore justified.

THE MEANING OF P_H

The concentration of any acid in a given solution is defined on the basis of its normality, namely, 1 gm. equivalent of hydrogen (not hydrogen ion) per liter. The concentration of ionic hydrogen is defined on the same basis, namely, 1 gm. equivalent of hydrogen ion (not hydrogen) in one liter and is called normal hydrogen ion. The concentration of hydrogen ion in neutral water for example is $\frac{N}{10,000,000}$. The reason for the unwieldiness of this expression is evident and a more convenient method of referring to these extreme dilutions of hydrogen ion near the neutral point is demanded. Therefore, using the N/1 solution as the basis of comparison, by referring to the number of times which a given solution is tenfold more dilute than N/1, the expression indicating its concentration is much shortened, and yet quite as significant for purposes of comparing with other concentration. This number Sørensen called P_H .

In accordance with this, then, the P_H of an N/100 hydrogen ion solution is 2, namely, 2 tenfold dilutions of a N/1 solution; of N/10,000 it is 4; N/10,000,000 it is 7, etc.

Obviously, the P_H of a solution is simply the logarithm of its dilution, namely, $\log V$.²

Measuring the P_H of a Solution: With due consideration for saturation with hydrogen gas and other appropriate steps in the technic of a determination, uniting two solutions of N/1 and N/10 through a U-tube containing strong KCl, and then joining the platinum electrodes, immersed in the solutions to the terminals of a voltmeter, one may prove that the platinum electrode in the more dilute solution is 58 millivolts less positive than the other. By keeping the dilution in one vessel at N/1 (normal hydrogen electrode) and increasing the dilution in the other by tenfold each time, it will be seen that for each tenfold increase in the dilution in this vessel, an additional increase of 58 millivolts is registered by the voltmeter. Therefore, if the P_H of any given solution is unknown, the voltage developed by it, divided by 58 millivolts, namely, $\frac{\text{E. M. F.}}{58}$, gives the number of its tenfold dilutions, namely, its P_H .

Therefore $\frac{\text{E. M. F.}}{58} = P_H = \log V = \log \frac{1}{C}$. The formula $P_H = \frac{\text{E. M. F.}}{58}$ is used provided one of the solutions is kept at N/1 (normal hydrogen electrode) as indicated. In practice, however, the normal calomel electrode

² Jour. Biol. Chem., 1915, 23, p. 475.

³ Since dilution (V) is the reverse of (C), i. e., concentration, the reverse of (C), i. e., $\left(\frac{1}{C}\right)$ is commonly used in place of (V) whence the expression $\log \frac{1}{C}$. But the objection to the expression $\log \frac{1}{C}$ is that it is easier to think of the logarithm of a whole number, $\log V$, than of the logarithm of the reciprocal of a fraction.

instead of the normal hydrogen electrode is used, and since it is 280 millivolts more positive than the normal hydrogen electrode, 280 must be subtracted from the E. M. F. The equation then becomes $P_H = \frac{E. M. F. - 280}{58}$. The element of temperature also enters into the equation in very accurate work, but between the temperature of 20 and 30 C., no correction will be necessary for readings accurate to 1 millivolt. The relation of temperature to the other factors is expressed in the following equations $P_H = \frac{E. M. F. - 0.28}{0.000198 \times T}$, in which T = the absolute temperature.

THE HYDROGEN ELECTRODE

Clark⁴ maintains that the old method of bubbling hydrogen gas through a culture whose hydrogen ion concentration is to be determined is useless. From four to twenty-four hours are necessary to attain saturation with the hydrogen, and consequently important changes in reaction by this time have occurred. Hasselbach,⁵ however, showed that if the culture fluid is confined in a vessel filled with H gas, saturation is very quickly attained by shaking the solution and gas together.

Then Acree and Myers⁶ showed that if complete saturation is to be obtained the containing vessel must have no dead spaces in its interior, namely, entrance or exit tubes, where portions of the fluid or gas can hide and thus prevent the remainder of the contents from continuing at equilibrium after the shaking process is stopped.

It is known that when two dissimilar solutions are brought into contact with each other, a small E. M. F. is generated at the zone of contact. Since this E. M. F. would cause a slight error* in the reading of the voltage developed at the electrodes themselves, the solution in the hydrogen electrode vessel after saturation with hydrogen gas is, in practice, brought into contact with the solution in the calomel electrode by means of a connecting column of KCl (about 3N). Cummings and Gilchrist⁷ have shown that this contact should be made in such a way that the solution saturated with hydrogen lies on the connecting solution of KCl in a zone whose area is not too small.

The electrode vessel here described was designed with these various suggestions in mind. Its parts are assembled as follows: A test tube of about 1 inch diameter is cut off at both ends, making a cylinder of about 3 inches long of the middle portion. Both ends should be burnished, and then properly fitted with 2-hole rubber stoppers. One of these stoppers is fitted with two L-tubes bent as illustrated, and sealed at their inner ends. A pinhole is made at each of these inner ends about $\frac{1}{8}$ inch from the tip. This is most easily done while the ends are still hot following the sealing process, by boring a red hot platinum needle through the wall of the glass tube, withdrawing, and then, by means of a file, breaking off the thread of glass which follows the needle, and filing the surface around the pinhole until smooth. One of these

* To measure the extent of this error, one may show by the hydrogen electrode apparatus that the chain $H_2|N/10HCl|KCl|N/100HCl|H_2$ develops an E. M. F. of 0.058 volts (since one solution has a tenfold greater concentration of hydrogen ion than the other); whereas, the chain $H_2|N/10HCl|N/100HCl|H_2$ develops only 0.019 volt. In other words, the E. M. F. developed at the zone of contact between the N/10 and N/100 solutions is 0.058-0.019 or 0.039 volts, and acts in opposition to the E. M. F. developed by the two electrodes, thereby reducing their voltage from 0.058 volts to 0.019 volts. This error of 0.039 volts, or 39 mv., is obviously too large to ignore in practice, and is due, as stated above, to the potential of the contact zone opposing the potential of the two electrodes.

⁴ Jour. Biol. Chem., 1915, 23, p. 475.

⁵ Biochem. Ztschr., 1911, 30, p. 317.

⁶ Am. Chem. Jour., 1913, 50, p. 396.

⁷ Tr. Faraday Soc., 1913, 19, p. 174.

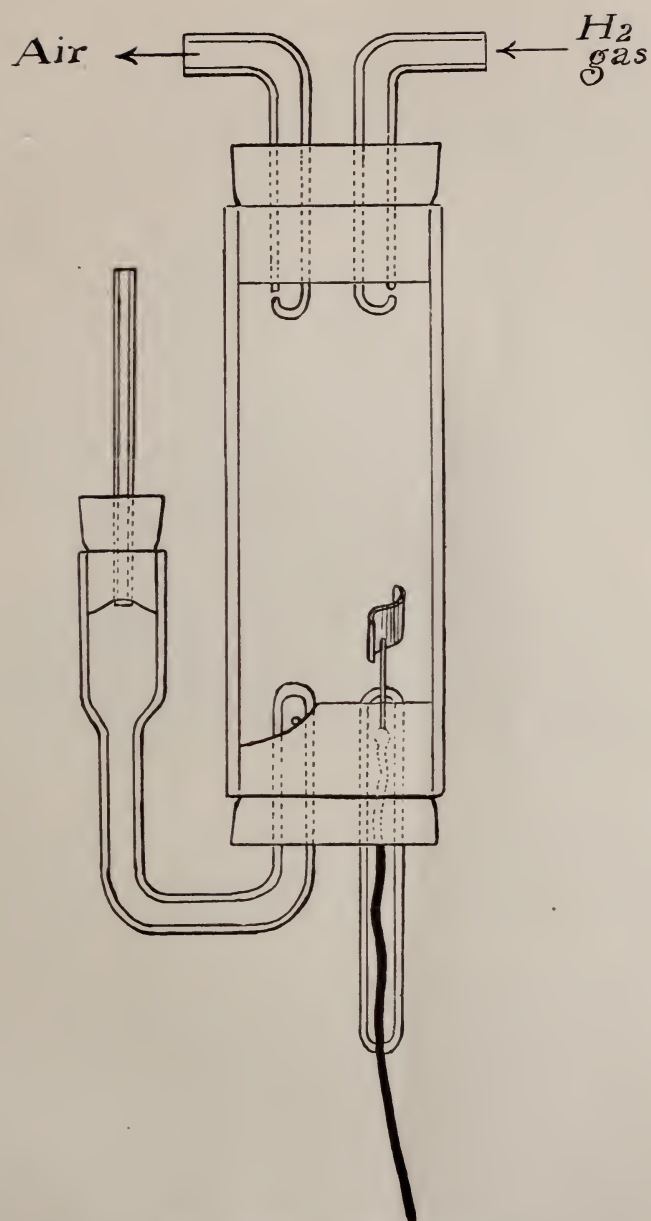


Fig. 1.—Diagram of hydrogen electrode.

pinholes, when the tubes are pushed in beyond the body of the stopper, serves as an entrance for hydrogen, the other as an exit for the air which is to be displaced.

An oblique slice is cut from the inner end of the other stopper as illustrated. This hole is fitted with the J-tube which will be used to contain the 3 N KCl solution (for connecting the solution saturated with H gas with the solution in the calomel electrode). The other hole receives the glass tube through which passes the copper wire to the platinum electrode. The J tube also has a pinhole near its tip, as illustrated, which should be rather large.

The platinum electrode is made by heating a bit of platinum plate and platinum wire to white heat on some smooth surface and then welding them together with a hot hammer. The platinum wire is then fused to the copper wire by holding the latter with some nonconducting substance, as cork, and plunging into a blow pipe or good bunsen flame until its tip softens to a small drop of molten copper, and then bringing the free tip of the platinum wire into the flame until it just touches the molten copper. The operation must be done quickly else a large ball of molten copper forms on the end of the wire. The copper wire is then placed within a short piece of glass tubing and the end of the latter sealed about the platinum wire portion as illustrated.

The large end of the J tube is to be closed by a small rubber stopper the smaller end of which is cut as illustrated, and through which passes a piece of open capillary glass tubing. The electrode vessel is then complete.

THE DETERMINATION

The purpose of the operation, when using the type of electrode vessel here described, is to fill the J-tube with the 3N KCl connecting solution, place in the vessel some of the solution to be tested, displace the air with H_2 gas, shake until the solution is saturated, make electrolytic contact between the latter and the calomel electrode, measure the E. M. F. developed, and from this, calculate the P_H of the solution.

The details of these various steps follow: Remove the small stopper with open capillary tubing from the end of the J-tube. Turn the J-tube so that the pin-hole near the tip of its other end appears. Remove the rubber stopper with the two L tubes from the vessel, and with a pipet run in to the J-tube some strong KCl solution (about 3N) until all the air is driven out by way of the pin-hole. Now turn the J-tube back again through about 90 degrees so that the pin-hole disappears. The KCl solution is thus held in the J-tube by the pressure of the rubber stopper over the pin-hole. Continue to run in KCl solution into the J-tube up to within 1 cm. of the top. Replace the small rubber stopper in such a way that the air bubble and excess of KCl solution will escape through the open capillary tube.

Wash out the vessel with some of the solution to be tested, then use enough of the latter to just cover the top edge of the platinum electrode. Replace the rubber stopper, having previously pushed in the L-tubes until their pin-holes are just visible. Now run in a slow stream of pure hydrogen gas by way of the L-tubes, for about 30 seconds, or until the air is all driven out by way of the other L-tube. Shut off the flow of hydrogen gas, gently withdraw the air exit L-tube just far enough to effectually close the pin-hole by the pressure of the rubber stopper, then in a similar way withdraw the other L-tube. We now have the solution to be tested in an atmosphere of hydrogen gas, and enclosed in a space which has no side pockets or "dead" spaces into which fluid or gas can hide and thus fail to reach equilibrium with the rest of the vessel contents.

After shaking the vessel for about one minute the small rubber stopper and capillary tube are withdrawn, and the J-tube again turned back far enough for the pin-hole to be seen. If this is done slowly, the KCl solution will be seen to flow in and quietly settle in a layer in the slight depression where the oblique slice of the rubber stopper was cut away. This then affords a wide zone of contact between the KCl solution and the solution to be tested. The vessel is now ready to be connected to the calomel electrode. This is done by simply adjusting the latter so that the tip of its connecting tube is dipped into the KCl solution in the J-tube.

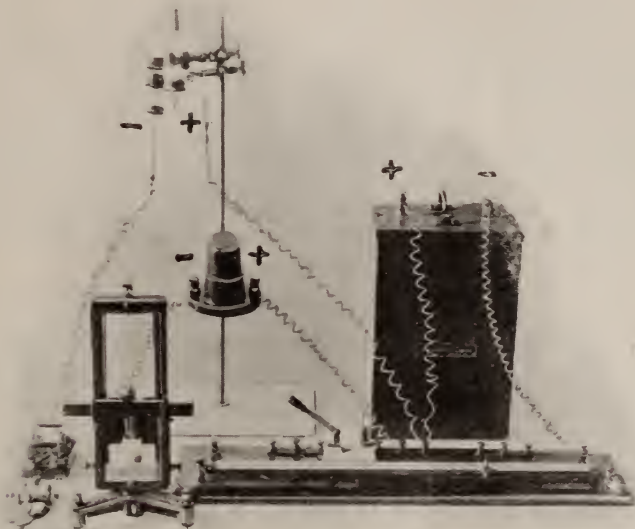


Fig. 2.—Photograph of apparatus for making hydrogen ion concentration determinations: Wheatstone bridge, Harvard switch, telegraph key, galvanometer, Weston standard cell, 2-volt lead accumulator, normal calomel electrode and hydrogen electrode. The positive and negative elements are indicated, and their connections easily traced. The bridge is shortened by laying on the 50 cm. scale only that section of the bridge wire commonly used in measurements made on biologic fluids, namely, the sections falling between 400 and 900 mm. from the positive end of the bridge.

It is desirable, if not necessary, to test the uniformity of the platinum electrodes after these have been platinized. For this, the author uses a vessel like the one already described, but large enough so that the rubber stopper has holes for three or four platinum electrodes instead of one. Once the platinum black on each electrode has become saturated with hydrogen gas the voltage developed by each should be identical. Differences are readily detected if present, and will probably be due to unclean platinum wire or plate. When properly platinized, the color of the electrode is a rich velvet black, but with use changes to brown, when it should be replatinized. The first determination made with a freshly platinized electrode will require several minutes more than subsequent ones for the reason that more time is required for the platinum black to become saturated with the hydrogen gas.

From a preceding paragraph it is seen that as the ionic dilution of a given solution is increased ten times, say from $N/1$ to $N/10$, its P_H is increased by 1, and its voltage by 0.058 volts. Therefore 0.058 volts or 58 millivolts = 1 P_H . Then 1 mv. = 0.02 P_H (about). An error, then, of 1 mm. in our reading would mean an error of 0.02 of a P_H . The apparatus is easily accurate to 0.5 mv. or 0.01 P_H . The author has found it desirable to combine the principles of the colorimetric method with the gas chain method, in those instances where large numbers of determinations are to be made as in bacterial cultures. This is done by taking a portion (2 cc) of each solution to be tested, diluting with 4 cc of distilled water, and after arranging the tubes in about three groups with reference to their degree of turbidity, placing each group in separate rows in test tube racks. Equal amounts of some indicator, with a range of color change adapted to the probably "acidities" of the solutions (one of those devised and recommended by Clark and Lubs⁸) is placed in each tube and shaken. In each group the colors developed enable one at once to collect the tubes into subgroups of similar colors. One specimen of each subgroup when tested with the hydrogen electrode, then gives the H-ion concentration of the entire subgroup, thus obviating the necessity of preparing standard solutions for color comparisons. In one series of determinations on about 80 cultures, it was found necessary when using this method to make actual measurements on only 6 specimens of the entire lot.

SUMMARY

A new apparatus for determining the dilution of hydrogen ion in bacterial cultures and other fluids is described. As compared with the colorimetric method, the apparatus here described has wider application, is more accurate, less cumbersome, and only slightly less rapid.

The hydrogen electrode vessel described, was designed with two objects, chiefly, in mind: (1) to provide a vessel accurate at least to 0.01 P_H and (2) to provide a vessel giving rapid saturation with hydrogen gas, and yet one which is easily constructed.

A rapid and labor saving technic combining the indicator, and the gas-chain methods is described, which obviates the difficult task of preparing standard solution for the former method, and of making needless repetitions by the latter.

⁸ Jour. Infect. Dis., 1915, 17, p. 160.

FROZEN COMPLEMENT IN THE WASSERMANN REACTION

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Without giving any details, Moledetzky says frozen complement retains its strength indefinitely. As complement usually deteriorates within 3 days it is the most expensive ingredient in the Wassermann reaction, and as the time for standardization is short a method by which complement can be preserved is very desirable. Other methods having been found unsatisfactory frozen complement was studied.

TEST 1

Complement serums 1, 2, 3, 5, 6 and 7, each composed of the pooled serums of 3 guinea-pigs, were divided into two portions, A and B. Portion A of each number was tested on 4 human serums within 24 hours after having been secured and without having been frozen. Portion B of each number was frozen within 24 hours after having been secured, it was kept frozen and at intervals of a week portion B of each number was tested on the same human serums that had been used in testing portion A. Each mixture of complement was kept under observation for a period of 4 weeks, at the end of which time the human serums were again tested with fresh complements 4 and 8 in order to detect any change in the human serums should there be such.

Table 1 shows the results obtained with mixed frozen complements. Frozen complement a week old usually was fixed a little better than was the fresh complement of the same number. With frozen complement two weeks old the results were almost identical with those obtained with the fresh complement. After two weeks fixability and hemolysis became poorer which was not due to changes in the human serums as is shown in the tests with control complements 4 and 8. Complements 5, 6 and 7 deteriorated more rapidly than did complements 1, 2 and 3, and was brought about by mild weather.

TEST 2

Complements 9, 10, 11, 13, 14 and 15 were unmixed, each was composed of the serum from one guinea-pig. Each complement serum was divided into two portions, A and B. Portion A of each number was tested on four human

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¹ Jour. Am. Med. Assn., 1918, 71, p. 968.

TABLE 1
MIXED FROZEN COMPLEMENT TESTED AT INTERVALS OF A WEEK

Number of Complement	Portions A-Fresh B-Frozen	Age, Weeks	Number of Serum	Dilution of Amboceptor	Readings*						Results
					Antigen Tubes			Control Tubes			
					1	2	3	1'	2'	3'	
1	A	0	1 2 3 4	1:400	tr ± 0 +	0 0 0 +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 8+ Strongly positive, 6+ Strongly positive, 5+ Negative, —
1	B	1	1 2 3 4	1:400	0 ± + +	0 0 0 +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 10+ Strongly positive, 6+ Strongly positive, 5+ Negative, —
1	B	2	1 2 3 4	1:400	0 tr + +	0 0 tr +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 10+ Strongly positive, 8+ Strongly positive, 4+ Negative, —
1	B	3	1 2 3 4	1:400	tr ± + +	0 0 tr +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 8+ Strongly positive, 6+ Strongly positive, 4+ Negative, —
1	B	4	1 2 3 4	1:400	tr + + +	0 tr tr +	0 0 0 tr	+	+	tr tr tr tr	Strongly positive, 8+ Strongly positive, 3+ Strongly positive, 3+ Negative, —
4	A	0	1 2 3 4	1:400	tr ± + +	0 0 0 +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 8+ Strongly positive, 6+ Strongly positive, 5+ Negative, —
2	A	0	5 6 7 8	1:400	+	± ± tr +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 3+ Strongly positive, 3+ Strongly positive, 4+ Negative, —
2	B	1	5 6 7 8	1:400	+	tr ± 0 +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 4+ Strongly positive, 3+ Strongly positive, 5+ Negative, —
2	B	2	5 6 7 8	1:400	+	± + tr +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 3+ Strongly positive, 3+ Strongly positive, 4+ Negative, —
2	B	3	5 6 7 8	1:400	+	± + ± +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 3+ Moderately positive, 2+ Strongly positive, 3+ Negative, —
2	B	4	5 6 7 8	1:400	+	± + tr +	0 0 0 tr	+	+	tr tr tr tr	Moderately positive, 2+ Moderately positive, 2+ Strongly positive, 3+ Negative, —
4	A	0	5 6 7 8	1:400	+	± + tr +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 3+ Strongly positive, 3+ Strongly positive, 4+ Negative, —
3	A	0	9 10 11 12	1:400	+	tr + + +	0 0 0 ±	+	+	± ± ± ±	Strongly positive, 4+ Strongly positive, 5+ Strongly positive, 5+ Negative, —

* Explanation: 0 = no hemolysis; tr (trace) = hemolysis up to 50%; ± = hemolysis between 50% and 100%; + = complete hemolysis.

TABLE 1—Continued
MIXED FROZEN COMPLEMENT TESTED AT INTERVALS OF A WEEK

Number of Complement	Portions A-Fresh B-Frozen	Age Weeks	Number of Serum	Dilution of Amboceptor	Readings						Results
					Antigen Tubes			Control Tubes			
					1	2	3	1'	2'	3'	
3	B	1	9	1:400	+	0	0	+	+	±	Strongly positive, 5+ Strongly positive, 6+ Strongly positive, 6+ Negative, —
			10		±	0	0	+	+	±	
			11		±	0	0	+	+	±	
			12		+	+	±	+	+	±	
3	B	2	9	1:400	+	0	0	+	+	tr	Strongly positive, 4+ Strongly positive, 6+ Strongly positive, 6+ Negative, —
			10		tr	0	0	+	+	tr	
			11		tr	0	0	+	+	tr	
			12		+	+	±	+	+	±	
3	B	3	9	1:400	+	tr	0	+	+	tr	Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 5+ Negative, —
			10		+	0	0	+	+	tr	
			11		±	0	0	+	+	tr	
			12		+	+	tr	+	+	tr	
3	B	4	9	1:400	+	0	0	+	+	0	Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 4+ Negative, —
			10		±	0	0	+	+	0	
			11		±	0	0	+	+	0	
			12		+	+	tr	+	+	tr	
4	A	0	9	1:400	+	tr	0	+	+	±	Strongly positive, 4+ Strongly positive, 6+ Strongly positive, 5+ Negative, —
			10		±	0	0	+	+	±	
			11		±	0	0	+	+	±	
			12		+	+	±	+	+	±	
5	A	0	13	1:400	+	±	0	+	+	±	Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 4+ Negative, —
			14		+	tr	0	+	+	±	
			15		+	tr	0	+	+	±	
			16		+	+	±	+	+	±	
5	B	1	13	1:400	+	tr	0	+	+	±	Strongly positive, 4+ Strongly positive, 5+ Strongly positive, 5+ Negative, —
			14		+	0	0	+	+	±	
			15		+	0	0	+	+	±	
			16		+	+	±	+	+	±	
5	B	2	13	1:400	+	tr	0	+	+	tr	Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 4+ Negative, —
			14		+	0	0	+	+	tr	
			15		+	0	0	+	+	tr	
			16		+	+	tr	+	+	tr	
5	B	3	13	1:400	tr	0	0	+	±	0	Strongly positive, 4+ Strongly positive, 4+ Strongly positive, 4+ Negative, —
			14		0	0	0	+	tr	0	
			15		0	0	0	+	tr	0	
			16		+	tr	0	+	tr	0	
5	B	4	13	1:400	±	0	0	+	tr	0	Moderately positive, 2+ Weakly positive, 1+ Unfit Unfit
			14		tr	0	0	±	0	0	
			15		tr	0	0	tr	tr	0	
			16		+	tr	0	±	tr	0	
8	A	0	13	1:400	+	tr	0	+	+	±	Strongly positive, 4+ Strongly positive, 5+ Strongly positive, 6+ Negative, —
			14		+	0	0	+	+	±	
			15		±	0	0	+	+	±	
			16		+	+	±	+	+	±	
6	A	0	17	1:400	tr	0	0	+	+	±	Strongly positive, 8+ Strongly positive, 6+ Strongly positive, 8+ Negative, —
			18		±	0	0	+	+	±	
			19		tr	0	0	+	+	±	
			20		+	+	±	+	+	±	
6	B	1	17	1:400	0	0	0	+	+	±	Strongly positive, 10+ Strongly positive, 8+ Strongly positive, 10+ Negative, —
			18		tr	0	0	+	+	±	
			19		0	0	0	+	+	±	
			20		+	+	±	+	+	±	

TABLE 1—Continued
MIXED FROZEN COMPLEMENT TESTED AT INTERVALS OF A WEEK

Number of Complement	Portions A—Fresh B—Frozen	Age, Weeks	Number of Serum	Dilution of Amboceptor	Readings						Results
					Antigen Tubes			Control Tubes			
					1	2	3	1'	2'	3'	
6	B	2	17	1:400	0	0	0	+	+	0	Strongly positive, 6+ Strongly positive, 6+ Strongly positive, 6+ Negative,
			18		0	0	0	+	+	0	
			19		0	0	0	+	+	0	
			20		+	+	0	+	+	0	
6	B	3	17	1:400	0	0	0	±	tr	0	Unfit Unfit Unfit Negative,
			18		0	0	0	±	tr	0	
			19		0	0	0	tr	tr	0	
			20		+	tr	0	+	tr	0	
6	B	4	17	1:400	0	0	0	tr	tr	0	Unfit Unfit Unfit Unfit
			18		0	0	0	tr	tr	0	
			19		0	0	0	tr	tr	0	
			20		±	tr	0	±	tr	0	
8	A	0	17	1:400	0	0	0	+	+	±	Strongly positive, 10+ Strongly positive, 8+ Strongly positive, 10+ Negative,
			18		tr	0	0	+	+	±	
			19		0	0	0	+	+	±	
			20		+	+	±	+	+	±	
7	A	0	21	1:400	+	tr	0	+	+	±	Strongly positive, 4+ Strongly positive, 4+ Strongly positive, 3+ Negative,
			22		+	tr	0	+	+	±	
			23		+	±	0	+	+	±	
			24		+	+	±	+	+	±	
7	B	1	21	1:400	+	0	0	+	+	±	Strongly positive, 5+ Strongly positive, 5+ Strongly positive, 4+ Negative,
			22		+	0	0	+	+	±	
			23		+	tr	0	+	+	±	
			24		+	+	±	+	+	±	
7	B	2	21	1:400	tr	0	0	+	+	0	Strongly positive, 5+ Strongly positive, 4+ Strongly positive, 3+ Negative,
			22		±	0	0	+	+	0	
			23		+	0	0	+	+	0	
			24		+	+	0	+	+	0	
7	B	3	21	1:400	+	0	0	+	±	0	Moderately positive, 2+ Moderately positive, 2+ Moderately positive, 2+ Negative,
			22		+	0	0	+	±	0	
			23		+	0	0	+	±	0	
			24		+	±	0	+	±	0	
7	B	4	21	1:400	±	0	0	+	tr	0	Moderately positive, 2+ Strongly positive, 3+ Strongly positive, 3+ Negative,
			22		±	0	0	+	±	0	
			23		+	0	0	+	±	0	
			24		+	tr	0	+	tr	0	
8	A	0	21	1:400	+	0	0	+	+	±	Strongly positive, 5+ Strongly positive, 4+ Strongly positive, 4+ Negative,
			22		+	tr	0	+	+	±	
			23		+	tr	0	+	+	±	
			24		+	+	±	+	+	±	

serums while fresh and portion B of each number was frozen, was kept frozen and was tested on the same human serums at intervals of a week for 4 weeks. At the end of 4 weeks the human serums were tested with fresh control complements (Nos. 12 and 16) in order to detect any changes in the human serums.

The results obtained with unmixing guinea-pig complements are shown in table 2. Frozen complement one week old frequently gave stronger positive results than did the same complement while fresh. Complement that had been kept frozen for 2 weeks gave results that

TABLE 2
 UNMIXED FROZEN COMPLEMENT TESTED AT INTERVALS OF A WEEK

Number of Complement	Portions A-Fresh B-Frozen	Age, Weeks	Number of Serum	Dilution of Amboceptor	Readings*						Results
					Antigen Tubes			Control Tubes			
					1	2	3	1'	2'	3'	
9	A	0	25	1:400	+	±	0	+	+	tr	Moderately positive, 2+ Strongly positive, 3+ Strongly positive, 3+ Negative, —
			26		+	tr	0	+	+	tr	
			27		+	tr	0	+	+	tr	
			28		+	+	tr	+	+	tr	
9	B	1	25	1:400	+	tr	0	+	+	0	Moderately positive, 2+ Strongly positive, 3+ Strongly positive, 3+ Negative, —
			26		+	tr	0	+	+	tr	
			27		+	tr	0	+	+	tr	
			28		+	+	tr	+	+	tr	
9	B	2	25	1:400	+	tr	0	+	+	0	Moderately positive, 2+ Strongly positive, 3+ Moderately positive, 2+ Negative, —
			26		+	tr	0	+	+	tr	
			27		+	±	0	+	+	tr	
			28		+	+	tr	+	+	tr	
9	B	3	25	1:400	+	tr	0	+	±	0	Weakly positive, 1+ Moderately positive, 2+ Moderately positive, 2+ Negative, —
			26		+	tr	0	+	+	0	
			27		+	tr	0	+	+	0	
			28		+	+	0	+	+	0	
9	B	4	25	1:400	+	tr	0	+	±	0	Weakly positive, 1+ Moderately positive, 2+ Moderately positive, 2+ Negative, —
			26		+	tr	0	+	+	0	
			27		+	tr	0	+	+	0	
			28		+	+	0	+	+	0	
12	A	0	25	1:400	+	+	0	+	+	±	Moderately positive, 2+ Strongly positive, 4+ Strongly positive, 3+ Negative, —
			26		+	tr	0	+	+	±	
			27		+	±	0	+	+	±	
			28		+	+	±	+	+	±	
10	A	0	29	1:400	+	±	0	+	+	tr	Moderately positive, 2+ Strongly positive, 4+ Strongly positive, 3+ Negative, —
			30		+	tr	0	+	+	±	
			31		+	±	0	+	+	±	
			32		+	+	±	+	+	±	
10	B	1	29	1:400	+	tr	0	+	+	tr	Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 4+ Negative, —
			30		+	tr	0	+	+	±	
			31		+	tr	0	+	+	±	
			32		+	+	±	+	+	±	
10	B	2	29	1:400	+	±	0	+	+	tr	Moderately positive, 2+ Strongly positive, 4+ Strongly positive, 3+ Negative, —
			30		+	tr	0	+	+	±	
			31		+	±	0	+	+	±	
			32		+	+	±	+	+	±	
10	B	3	29	1:400	+	tr	0	+	+	0	Moderately positive, 2+ Strongly positive, 3+ Strongly positive, 3+ Negative, —
			30		+	tr	0	+	+	tr	
			31		+	tr	0	+	+	tr	
			32		+	+	tr	+	+	tr	
10	B	4	29	1:400	+	tr	0	+	±	0	Weakly positive, 1+ Moderately positive, 2+ Moderately positive, 2+ Negative, —
			30		+	tr	0	+	+	0	
			31		+	tr	0	+	+	0	
			32		+	+	tr	+	+	tr	
12	A	0	29	1:400	+	±	0	+	+	±	Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 3+ Negative, —
			30		+	tr	0	+	+	±	
			31		+	±	0	+	+	±	
			32		+	+	±	+	+	±	
11	A	0	33	1:400	+	+	0	+	+	±	Moderately positive, 2+ Strongly positive, 4+ Strongly positive, 3+ Negative, —
			34		+	tr	0	+	+	±	
			35		+	±	0	+	+	±	
			36		+	+	±	+	+	±	

* Explanation: 0 = no hemolysis; tr (trace) = hemolysis up to 50%; ± = hemolysis between 50% and 100%; + = complete hemolysis.

TABLE 2—Continued
 UNMIXED FROZEN COMPLEMENT TESTED AT INTERVALS OF A WEEK

Number of Complement	Portions A-Fresh B-Frozen	Age, Weeks	Number of Serum	Dilution of Amboceptor	Readings						Results	
					Antigen Tubes			Control Tubes				
					1	2	3	1'	2'	3'		
11	B	1	33	1:400	+	±	0	+	+	±	Strongly positive, 3+ Strongly positive, 4+ Strongly positive, 3+ Negative, —	
			34		+	tr	0	+	+	±		
			35		+	±	0	+	+	±		
			36		+	+	±	+	+	±		
11	B	2	33	1:400	+	+	0	+	+	±	Moderately positive, 2+ Strongly positive, 4+ Strongly positive, 3+ Negative, —	
			31		+	tr	0	+	+	±		
			35		+	tr	0	+	+	tr		
			36		+	+	±	+	+	±		
11	B	3	33	1:400	+	tr	0	+	+	0	Moderately positive, 2+ Moderately positive, 2+ Moderately positive, 2+ Negative, —	
			34		+	±	0	+	+	tr		
			35		+	tr	0	+	+	0		
			36		+	+	tr	+	+	tr		
11	B	4	33	1:400	+	0	0	+	tr	0	Weakly positive, 1+ Moderately positive, 2+ Moderately positive, 2+ Negative, —	
			34		+	0	0	+	±	0		
			35		+	tr	0	+	+	0		
			36		+	+	0	+	+	0		
12	A	0	33	1:400	+	+	0	+	+	±	Moderately positive, 2+ Strongly positive, 4+ Strongly positive, 3+ Negative, —	
			24		+	tr	0	+	+	±		
			35		+	±	0	+	+	±		
			36		+	+	±	+	+	±		
13	A	0	37	1:400	±	0	0	+	+	tr	Strongly positive, 5+ Strongly positive, 5+ Strongly positive, 5+ Negative, —	
			38		±	0	0	+	+	tr		
			39		±	0	0	+	+	tr		
			40		+	+	tr	+	+	tr		
13	B	1	37	1:400	tr	0	0	+	+	0	Strongly positive, 5+ Strongly positive, 6+ Strongly positive, 6+ Negative, —	
			38		tr	0	0	+	+	tr		
			39		tr	0	0	+	+	tr		
			40		+	+	tr	+	+	tr		
13	B	2	37	1:400	tr	0	0	+	+	0	Strongly positive, 5+ Strongly positive, 5+ Strongly positive, 6+ Negative, —	
			38		±	0	0	+	+	tr		
			39		tr	0	0	+	+	tr		
			40		+	+	tr	+	+	tr		
13	B	3	37	1:400	±	0	0	+	+	0	Strongly positive, 4+ Strongly positive, 5+ Strongly positive, 6+ Negative, —	
			38		tr	0	0	+	+	0		
			39		0	0	0	+	+	0		
			40		+	+	tr	+	+	tr		
13	B	4	37	1:400	±	0	0	+	±	0	Strongly positive, 3+ Strongly positive, 3+ Strongly positive, 4+ Negative, —	
			38		±	0	0	+	±	0		
			39		tr	0	0	+	±	0		
			40		+	±	0	+	±	0		
16	A	0	37	1:400	+	0	0	+	+	±	Strongly positive, 5+ Strongly positive, 6+ Strongly positive, 6+ Negative, —	
			38		±	0	0	+	+	±		
			39		±	0	0	+	+	±		
			40		+	+	±	+	+	±		
14	A	0	41	1:400	+	tr	0	+	+	±	Strongly positive, 4+ Strongly positive, 5+ Strongly positive, 4+ Negative, —	
			42		+	0	0	+	+	±		
			43		+	tr	0	+	+	±		
			44		+	+	±	+	+	±		
14	B	1	41	1:400	+	tr	0	+	+	±	Strongly positive, 4+ Strongly positive, 5+ Strongly positive, 4+ Negative, —	
			42		+	0	0	+	+	±		
			43		+	tr	0	+	+	±		
			44		+	+	±	+	+	±		

TABLE 2—Continued
UNMIXED FROZEN COMPLEMENT TESTED AT INTERVALS OF A WEEK

Number of Comple- ment	Portions A—Fresh B—Frozen	Age, Weeks	Number of Serum	Dilu- tion of Ambo- ceptor	Readings						Results		
					Antigen Tubes			Control Tubes					
					1	2	3	1'	2'	3'			
14	B	2	41	1:400	+	tr	0	+	+	±	Strongly positive, Strongly positive, Strongly positive, Negative,	4+	
			42		+	tr	0	+	+	±		4+	
			43		+	tr	0	+	+	±		4+	
			44		+	+	±	+	+	±		—	
14	B	3	41	1:400	+	tr	0	+	+	tr	Strongly positive, Strongly positive, Strongly positive, Negative,	3+	
			42		+	0	0	+	+	tr		4+	
			43		+	tr	0	+	+	tr		3+	
			44		+	+	tr	+	+	tr		—	
14	B	4	41	1:400	+	0	0	+	+	0	Strongly positive, Strongly positive, Strongly positive, Negative,	3+	
			42		±	0	0	+	+	0		4+	
			43		+	0	0	+	+	0		3+	
			44		+	+	0	+	+	0		—	
16	A	0	41	1:400	+	tr	0	+	+	±	Strongly positive, Strongly positive, Strongly positive, Negative,	4+	
			42		+	0	0	+	+	±		5+	
			43		+	0	0	+	+	±		5+	
			44		+	+	±	+	+	±		—	
15	A	0	45	1:400	±	0	0	+	+	±	Strongly positive, Strongly positive, Strongly positive, Negative,	6+	
			46		±	0	0	+	+	±		6+	
			47		+	0	0	+	+	±		5+	
			48		+	+	±	+	+	±		—	
15	B	1	45	1:400	tr	0	0	+	+	±	Strongly positive, Strongly positive, Strongly positive, Negative,	8+	
			46		±	0	0	+	+	±		6+	
			47		±	0	0	+	+	±		6+	
			48		+	+	±	+	+	±		—	
15	B	2	45	1:400	±	0	0	+	+	±	Strongly positive, Strongly positive, Strongly positive, Negative,	6+	
			46		±	0	0	+	+	±		6+	
			47		+	0	0	+	+	±		5+	
			48		+	+	±	+	+	±		—	
15	B	3	45	1:400	±	0	0	+	+	tr	Strongly positive, Strongly positive, Strongly positive, Negative,	5+	
			46		±	0	0	+	+	tr		5+	
			47		+	0	0	+	+	tr		4+	
			48		+	+	tr	+	+	tr		—	
15	B	4	45	1:400	±	0	0	+	+	0	Strongly positive, Strongly positive, Strongly positive, Negative,	4+	
			46		tr	0	0	+	+	0		5+	
			47		+	0	0	+	+	0		3+	
			48		+	+	0	+	+	0		—	
16	A	0	45	1:400	±	0	0	+	+	±	Strongly positive, Strongly positive, Strongly positive, Negative,	6+	
			46		±	0	0	+	+	±		6+	
			47		+	0	0	+	+	±		5+	
			48		+	+	±	+	+	±		—	

were almost identical with those given by the fresh complement. After the frozen complement was more than 2 weeks old fixability and hemolytic power gradually decreased; that this decrease in fixability and hemolytic power was not due to changes in the human serums tested is shown by the results given by control complements 12 and 16, which were nearly identical with the results obtained with the fresh complement 4 weeks previously.

SUMMARY

Frozen guinea-pig complement one week old, whether composed of the mixed serums of 3 guinea-pigs or of the serum from one guinea-pig usually gave a little stronger positive results with the Wassermann reaction than did the same complement while fresh. At the age of 2 weeks the frozen complement gave results that were nearly identical with the results obtained with the fresh complements. After 2 weeks the frozen complement gradually lost strength which seemed to be more rapid in mild weather than in very cold weather. (May 10, 1919.)

THE SELECTIVE INHIBITORY ACTION OF METHYLENE BLUE AND CERTAIN OTHER COMMON DYES ON THE GROWTH OF MENINGOCOCCI

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In an effort to demonstrate the presence of capsules in certain strains of meningococci it was observed that safranin added to suspensions of the bacteria caused agglutination and subsequent settling of the suspended organism. Further investigation of this phenomenon showed that agglutination and complete precipitation occurred in dilutions of 1:2,000 and partial agglutination in dilutions of 1:10,000. This was first suspected of being a specific phenomenon, but the action of safranin on suspensions of *B. typhosus* and *Streptococcus viridans* revealed a similar agglutination. Subsequently it was found that the dye added in like dilutions to infusion broth caused a flocculent coagulation of protein closely resembling what had been regarded as agglutination. It may be assumed, perhaps, that the agglutination of suspended organisms caused by safranin is really due to protein coagulation. A further study of the action of safranin on suspensions of meningococci showed inhibition of their growth at dilutions between 1:1,000 and 1:10,000. This led to an investigation of the behavior of certain other common dyes both on meningococci and other pathogenic organisms, and a comparison of their inhibitory action with that of a few of the common antiseptics.

All meningococci used were obtained from the spinal fluids of cases of epidemic cerebro-spinal meningitis among troops in France. Where the type of meningococcus is given it was established on the basis of the French classification, using immune type serum obtained from the Pasteur Institute. Meningococci were cultivated on Kinnicutt's coagulated sheep blood medium—defibrinated sheep blood, 2 or 3 parts; dextrose infusion broth (0.2 acid to phenolphthalein), 1 part. This is an admirable medium for meningococci. Growth usually takes place in 24 hours, the organisms forming circular, raised, shiny, grayish colonies of characteristic appearance. Meningococci cultivated on this medium retain their viability at incubator temperature for a month or more, when rubber stoppers are used to prevent evaporation and drying.

One per cent. solutions of the dyes were made in sterile distilled water using sterile glassware. The solutions were then arnoldized. From 1% solutions dilutions were made 10 times stronger than the ultimate dilutions desired. The ultimate dilutions were obtained by adding 9 parts of bacterial suspensions to 1 part of diluted dye. In the majority of experiments the series of dilutions were set up in small test tubes measuring approximately 6 by 1.2 cm. In these tubes 0.1 cc diluted dye and 0.9 cc suspensions were mixed. In some experiments larger volumes were employed, namely, 1 cc dye and 9 cc suspension.

Suspensions of approximately the same density were made in sterile normal salt solution from organisms grown on solid mediums.

The mixtures of dye and bacterial suspension were allowed to remain at incubator temperature for one hour. They were then planted by pouring the entire contents of the tubes into culture tubes of slanted solid medium, from which the water of condensation had been poured off. By tilting the culture tubes the dye suspension mixtures were flowed over the surface of the medium three times. They were then incubated in the upright

position without permitting dye suspension mixtures to come in contact with slanted surface again. Cultures were examined for growth after 24-48 hours.

A more desirable technic would have been to cultivate the organisms directly in fluid medium containing dyes at various dilutions, were it not for the aerobic habits of the meningococcus; growth in fluid medium being scant and capricious.

In each experiment the use of a control tube containing 1 part of distilled water (in place of dye) and 9 parts of bacterial suspension demonstrated that the organisms retained their viability under the conditions of the experiment.

Exper. 1.—Comparative inhibitory action of gentian violet, safranin, methylene blue, fuchsin, eosin and phenol on meningococcus, strain 4S, type "B." Suspensions made with 60 cc normal saline solution from three 42-hour cultures in Pasteur tubes. Dye suspension mixtures in incubator 1 hour and twenty minutes before planting.

Dilutions	Gentian Violet	Safranin	Methylene (Leitz) Blue	Fuchsin (Basic)	Eosin	Phenol
1: 1,000.....	—	—	—	±	+	+
1: 2,000.....	—	—	±	±	+	+
1: 4,000.....	—	+	±	+	+	+
1: 8,000.....	—	+	±	+	+	+
1: 10,000.....	—	+	+	+	+	+
1: 20,000.....	—	+	+	+	+	+
1: 40,000.....	—	+	+	+	+	+
1: 80,000.....	+	+	+	+	+	+
Control.....	+	+	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.

± indicates a very slight growth after 48 hours' incubation, recognizable only by aid of a hand lens.

— indicates no macroscopic growth after 48 hours' incubation.

Exper. 2.—Comparative inhibitory action of crystal violet, brilliant green, bismarck brown, methylene blue (American and French preparations), vital red and fluorescein on meningococcus strain 4S, type "B." Suspension made with about 60 cc normal saline from one 48-hour culture in Pasteur tube. Dye suspension mixture in incubator 1 hour and 15 minutes before planting.

Dilutions	Crystal Violet	Brilliant Green	Bismarck Brown	Methylene Blue (American)	Methylene Blue (French)	Vital Red	Fluorescein
1: 1,000.....	—	—	+	+
1: 2,000.....	—	—	—	—	—	+	+
1: 4,000.....	—	—	—	—	—	+	+
1: 8,000.....	—	—	—	—	+	+	+
1: 10,000.....	—	—	—	—	+	+	+
Control.....	+						

Examination of these results shows that at the dilutions employed several of the dyes had a marked inhibitory action on the growth of this strain of meningococcus, whereas others had little or none. Because of its relative lack of toxicity it was determined to investigate further the action of methylene blue.

Exper. 3.—Comparative inhibitory action of methylene blue on meningococci, types "A," "B" and "C," and various other pathogenic organisms. Suspensions

made in sterile saline and diluted to approximately the same density. All cultures except *B. typhosus* 30 hours old. *B. typhosus* culture 10 hours. Dye suspension mixtures in incubator 1 hour before planting.

Dilutions of Methylene Blue (Leitz)	Meningococcus Type "A"	Meningococcus Type "B"	Meningococcus Type "C"	Bacillus Typhosus	Bacillus Coli	Bacillus Dysenteriae	Bacillus Pyocyaneus	Staphylococcus Albus	Streptococcus Viridans	Streptococcus Hemolyticus	Diphtheria Bacillus	Pneumococcus Group III
1: 1,000.....	—	—	—	+	+	+	+	+	+	+	+	+
1: 2,000.....	—	—	—	++	++	++	++	++	++	++	++	++
1: 4,000.....	—	++	—	++	++	++	++	++	++	++	++	++
1: 8,000.....	—	++	+	++	++	++	++	++	++	++	++	++
1: 10,000.....	++	++	+	++	++	++	++	++	++	++	++	++
1: 20,000.....	++	++	+	++	++	++	++	++	++	++	++	++
1: 30,000.....	++	++	+	++	++	++	++	++	++	++	++	++
1: 40,000.....	++	++	+	++	++	++	++	++	++	++	++	++
1: 50,000.....	+	+	+	+	+	+	+	+	+	+	+	+
Control.....	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.

++ indicates a very slight growth after 48 hours' incubation, recognizable only by aid of a hand lens.

— indicates no macroscopic growth after 48 hours' incubation.

Under the conditions of this experiment inhibition of growth occurred in cultures of the three strains of meningococci alone and in none of the other pathogenic organisms.

To determine the behavior of methylene blue on an organism biologically related to meningococcus a strain of gonococcus was obtained in pure culture and comparative titrations made with it and two strains of meningococci.

Exper. 4.—Comparative inhibitory action on gonococcus and on meningococci strain 4S, type "A," and strain 31S, type undetermined. Suspensions of gonococcus made with 10 cc sterile saline from 3 Kinnicutt's coagulated sheep blood cultures, respectively, 48 hours, 4 and 5 days old. Suspensions of meningococci for each strain made with 60 cc sterile saline from 48-hour Pasteur tube culture. Dye suspension mixtures in incubator 1 hour and 15 minutes before planting.

Dilutions of Methylene Blue (French)	Gonococcus	Meningococcus 4S	Meningococcus 31S
1: 1,000.....	—	—	—
1: 2,000.....	—	—	—
1: 4,000.....	—	—	—
1: 8,000.....	—	—	+
1: 10,000.....	—	—	+
Control.....	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.

— indicates no macroscopic growth after 48 hours' incubation.

In these experiments the action of methylene blue on the gonococcus and meningococcus showed a correspondence which was at variance with its action on the other pathogenic organisms tried.

From the tables it will be observed that the dilution of methylene blue at which growth capacity of the meningococcus is lost is variable. Even with constant conditions of medium, age of culture and time of incubation of dye suspension mixtures, there are a number of variables which no doubt contribute to establish this point. An important and obvious one is the number of organisms present in a suspension. To test this the action of methylene blue on a suspension was compared with its action on one made by diluting the first suspension 10 times.

Exper. 5.—Comparative inhibitory action of methylene blue on meningococcus strain 1S, type "A," and on *B. typhosus* suspensions of varying density. Original suspensions made with 20 cc sterile saline from 79-hour culture of meningococcus and 20-hour *B. typhosus*. Original suspension diluted by 10. Dye suspension mixtures in incubator 1 hour before planting.

Dilutions of Methylene Blue (Leitz)	Meningococcus Suspension	Meningococcus Suspension Diluted x10	Bacillus Typhosus Suspension	Bacillus Typhosus Suspension Diluted x10
1: 10,000.....	—	—	+	+
1: 20,000.....	—	—	+	+
1: 30,000.....	+	—	+	+
1: 40,000.....	+	—	+	+
1: 50,000.....	+	—	+	+
1: 60,000.....	+	—	+	+
1: 70,000.....	+	+	+	+
1: 80,000.....	+	+	+	+
1: 90,000.....	+	+	+	+
1: 100,000.....	+	+	+	+
1: 150,000.....	+	+	+	+
1: 200,000.....	+	+	+	+
1: 300,000.....	+	+	+	+
Control.....	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.

— indicates no macroscopic growth after 48 hours' incubation.

A variation between 1:10,000 and 1:60,000 is seen here in the inhibitory point of methylene blue on the growth of this strain of meningococcus, depending on the number of organisms present in the suspension. With *B. typhosus* no inhibition occurred even in the dilute suspension.

To determine whether for a given suspension the inhibitory point of methylene blue is constant, an experiment was made using three strains of meningococci. The suspensions made from each were titrated in triplicate. An inhibitory point was found different for the three strains, but constant for each set of triplicates.

Exper. 6.—Comparative inhibitory action of methylene blue on strains of meningococci 4S, 30S and 31S, each one titrated in triplicate. Suspensions made with 60 cc sterile saline from one 48-hour Pasteur tube culture each of 4S and 31S and with 30 cc sterile saline from a similar culture of 30S. Dye suspension mixtures in incubator for 1 hour and 45 minutes.

Dilutions of Methylene Blue (French)	4S			30S			31S		
	1	2	3	1	2	3	1	2	3
1: 1,000.....	—	—	—	—	—	—	—	—	—
1: 2,000.....	—	—	—	±	±	±	—	—	—
1: 4,000.....	—	—	—	+	+	+	—	—	—
1: 8,000.....	+	+	+	+	+	+	—	—	±*
1: 10,000.....	+	+	+	+	+	+	—	—	—
Control.....	+	+	+	+	+	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.

± indicates a very slight growth after 48 hours' incubation, recognizable only by aid of a hand lens.

— indicates no macroscopic growth after 48 hours' incubation.

* In this tube after 48 hours' incubation 3 colonies appeared on the slanted surface.

Because of the difficulty in obtaining suspensions of different strains of meningococci with equal numbers of organisms present, it seemed impracticable to attempt to express the inhibitory action of methylene blue on the meningococcus in any terms equivalent to a phenol coefficient. For one particular strain, however, a comparative study of the action of methylene blue and certain common disinfectants seemed of interest.

Exper. 7.—Comparative inhibitory action of formaldehyd, mercuric chlorid, phenol and methylene blue on meningococcus strain 4S, type "B." Suspensions made from four 24-hour cultures in Pasteur tubes.

Dilutions	Methylene Blue (French)	Formalin	Mercuric Chlorid	Phenol
1: 1,000	—	—	—	+
1: 2,000	—	—	—	+
1: 4,000	+	+	—	+
1: 8,000	+	+	—	+
1: 10,000	+	+	—	+
Control		+		

+ indicates a macroscopic growth after 48 hours' incubation.

— indicates no macroscopic growth after 48 hours incubation (with phenol, inhibition occurred in a dilution of 1:100).

With this particular suspension the inhibitory point of methylene blue corresponded to that of a solution of formalin.

To investigate what influence the presence of protein had on the inhibitory action of methylene blue the following experiment was done.

Expt. 8.—Action of methylene blue in various dilutions on meningococcus in presence of leukocytes and native protein of spinal fluid.

About 60 cc of turbid spinal fluid from a fresh case of cerebrospinal meningitis was collected in a large test tube. Approximately $\frac{1}{3}$ of this was planted by pouring into a flat-sided Pasteur tube containing the coagulated sheep blood medium. This and the remainder of the spinal fluid were incubated. After 48 hours a heavy growth appeared on the coagulated sheep blood. A suspension was made from this using the rest of the incubated spinal fluid to suspend the growth, in place of normal saline. The whole was thoroughly agitated to insure an equal distribution of organisms and sedimented pus cells. It was then added to varying dilutions of methylene blue in the proportion of 9 cc suspension to 1 cc of dye. The dye suspension mixtures were incubated for 1 hour and then poured into the Pasteur tubes containing the medium.

The accompanying table shows the result of this experiment. The presence of the native protein of inflammatory spinal fluid apparently exerted no influence on the inhibitory action of methylene blue.

Dilution of Methylene Blue (Leitz) in Spinal Fluid	Meningococcus Strain 308. Type Not Determined
1 : 2,000.....	—
1 : 4,000.....	—
1 : 8,000.....	—
1 : 10,000.....	—
Control.....	+

+ indicates a macroscopic growth after 48 hours' incubation.

— indicates no macroscopic growth after 48 hours incubation.

SUMMARY AND DISCUSSION

Of dyes studied, the following were found to inhibit the growth of meningococci: gentian violet, crystal violet, brilliant green, bismarck brown, safranin, methylene blue. In contrast to the above, basic fuchsin, vital red, fluorescein and eosin had no inhibitory action.

The study of the comparative action of methylene blue on various types of meningococci and other pathogenic organisms showed that the growth of meningococci was inhibited at dilutions which failed to inhibit the growth of the other organisms with one exception.

The inhibitory action of methylene blue on the meningococcus and the gonococcus, biologically related organisms, was the same.

No fixed point of dilution has been established at which methylene blue inhibits the growth of different suspensions of meningococci. This varies with the number of viable organisms present in the suspensions.

For a given suspension, however, the inhibitory point is constant.

A study of the comparative action of methylene blue, formaldehyd, mercuric chlorid and phenol showed mercuric chlorid to exert the

most powerful action, phenol the least, while methylene blue and formaldehyd exerted an equal influence intermediate between the other two.

The presence of native protein of inflammatory cerebrospinal fluid did not interfere with the inhibitory action of methylene blue on the meningococci.

The selective inhibitory action of methylene blue on the growth of the meningococcus group of organisms may be of a specific chemical nature, or it may be nonspecific, and due to the fact that the meningococcus is a relatively frail organism requiring, when artificially cultivated, a fairly congenial environment. To determine the specificity of the reaction would require the use of a greater variety of structurally allied chemicals and the titration of each against a variety of bacterial suspensions.

This work was approached rather from the practical point of view and an inquiry into the nature of the inhibitory phenomenon has not been attempted.

Contact with any considerable number of cases of epidemic cerebrospinal meningitis emphasized the fact that serum treatment as commonly practiced leaves much to be desired. Under ideal conditions, that is, early treatment with an effective serum, results are of course of proved success. But under conditions of late diagnosis and treatment with serum obtained from presumably reliable sources, but often of little or no value, results are frequently discouraging.

The hope of finding a chemical therapeutic agent for the meningococcus group of infections is what prompted this study. Methylene blue may possibly fill the requirements. It inhibits the growth of meningococci in fairly high dilutions. It is relatively nontoxic, is said to be diffusible through the choroid plexus and to have a specific affinity for nerve tissue.

Application to *in vivo* conditions from experiments conducted *in vitro* must, of course, be made with caution.

THE DISSEMINATION AND DESTRUCTION OF TYPHOID BACILLI INJECTED INTRAVENOUSLY IN NORMAL AND IMMUNE RABBITS

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Typhoid fever, although one of the first diseases in which the causative factor, the typhoid bacillus, was found, is still among the diseases which rank high in yearly mortality and disability statistics. This is especially true in those places where the value of prophylactic immunization is not widely advertised, or where owing either to carelessness or ignorance, those attending typhoid cases or convalescents do not take proper precautions against infection from their patients. However, in those countries where vaccination is advised, or, as in the case of the American and British armies, compulsory, typhoid fever has dropped to a level where it is no longer considered a serious menace to the population. Next to the vaccination against smallpox, typhoid vaccination has proved the most successful in preventing disease, although the duration of the immunity is not as long as that conferred by smallpox vaccination. But in spite of our early knowledge of the localization and characteristic of the typhoid bacillus, and our present knowledge of the immunity either on recovery from the disease itself or by vaccination, the exact mechanism by which the body is able to protect itself and the manner in which it gets rid of the offending invaders, is still unknown. Could we but understand the exact nature of this mechanism in a disease such as typhoid where our knowledge of the organism, symptoms, and such are so clear, we might well be able to apply similar deductions to other diseases in which the cause is more or less obscure, and undoubtedly assist the body in its fight to overcome the abnormal conditions under which it is struggling to maintain its own.

The typhoid bacillus was first demonstrated, as is well known, in 1880. Eberth, Klebs, and Koch simultaneously reported the finding of small rods in the tissues of those dying of typhoid fever, and in a sufficient number of cases to warrant their suspicions that this was the causative factor of the disease. However, Eberth¹ is usually given credit for the discovery because of

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¹ Virchows Arch., 1880, 81, p. 58.

a slight priority in the appearance of his article. The organism was first grown on artificial culture medium by Gaffky² in 1884. Klebs, just previous to the article by Gaffky, had grown cultures of organisms from the tissues of cases succumbing to typhoid, but his description of his cultures is considerably at variance with the typhoid bacillus as it is generally pictured in later and more conclusive work. However, the chains of organisms which he describes may have been due either to some peculiarity in the medium used, or to the fact that organisms recently isolated from the host may have certain unusual appearances which they lose after artificial cultivation. In 1885 A. Pfeiffer³ isolated the bacillus from stools of typhoid patients and in 1886 Heuppe demonstrated the organisms in the urine. In 1886 Fraenkel and Simmonds⁴ isolated the organism from the blood of a cadaver, but in 1887 came the important discovery of Vilchur, who was successful in isolating typhoid from the blood of living patients, although his percentage of positive cultures was very small.

In 1891, Blackstein⁵ reported the important discovery that the bile of rabbits injected intravenously with living typhoid bacilli, harbored these organisms for as long as 109 days after inoculation. Fütterer⁶ had previously laid stress on the fact that typhoid bacilli were found in the bile of cases of fever coming to necropsy, a finding which disproved, to a certain extent, the former belief that the bile was of high antiseptic value. The report of Blackstein that he had isolated the bacillus from the gallbladder 109 days after inoculation, however, was of far greater importance since it opened up a new avenue for study of a disease which, at that time, was making great inroads on the population. The route by which the bacillus reached the gallbladder was immediately subject to investigation. The theory that it ascended from the intestine was the first to be brought forth, but this was soon cast aside in favor of two more logical theories, namely, (1) that the organism is brought to the liver by the portal circulation, and from there swept down with the bile into the gallbladder and thence into the intestine, or (2) that it is carried directly to the gallbladder by the blood vessels which supply it. The former theory has been supported by the experimental work of Doerr,⁷ Nichols,⁸ and others, while the latter theory has been substantiated by the work of J. Koch,⁹ Chirolanza,¹⁰ Forster and Kayser.¹¹ Chiari¹² thinks all three ways possible. But the work of Blumenthal¹³ and later investigators would seem to prove that the latter theories are the more logical. As examples of the work to substantiate these two theories we may give a brief outline of the experiments carried out by Doerr and Chirolanza. Doerr, for example, ligated the common duct of rabbits, after which they were given intravenous injections of living typhoid bacilli. Gallbladder infection was, in these animals, constant. If, on the other hand, the cystic duct was ligated, he could demonstrate no organisms in the gallbladder. Chirolanza found typhoid bacilli present in the gallbladder

² Mitt. a. d. Kais. Gesundheit, 1884, 2, p. 372.

³ Deutsch. med. Wchnschr., 1885, 12, p. 500.

⁴ Die Aetiologische Bedeutung des Typhus-bacillus, 1886.

⁵ Bull. Johns Hopkins Hosp., 1891, p. 96.

⁶ München. med. Wchnschr., 1888, 35, p. 315.

⁷ Centralbl. f. Bakteriöl., 1905, 39, p. 624.

⁸ Jour. Exper. Med., 1914, 20, p. 573.

⁹ Ztschr. f. Hyg. u. Infektionskr., 1909, 62, p. 1.

¹⁰ Ztschr. f. Hyg. u. Infektionskr., 1900, 62, p. 11.

¹¹ München. med. Wchnschr., 1905, 52, p. 1423.

¹² Centralbl. f. Bakteriöl., 1894, 15, p. 648.

¹³ Centralbl. f. Bakteriöl., I. O., 1910, 28, p. 1314.

when the cystic duct was closed. He pointed out, furthermore, that the gallbladder is well supplied with blood vessels and that typhoid bacilli were sometimes found in the folds of the mucosa near the capillaries, whereas the bile itself might be sterile. J. Koch was also able to demonstrate these clumps of organisms in cut sections. No matter what theory may be assumed to be correct, the fact still remains that typhoid bacilli can be isolated from the gallbladder for a long period of time after injection. For example, Chirolanza demonstrated them 58 days after injection, Blackstein—109 days, Morgan¹⁴—4 months, and Uhlenhuth and Messerschmidt¹⁵—6 months. Doerr was able to isolate it from the interstitial mucosa 120 days after inoculation, which probably meant a locus of infection in the gallbladder, from which organisms were being swept down with the bile.

The establishing of "carrier" rabbits has not been successful in the hands of all investigators. Blackstein, for instance, could obtain only about 50 per cent. of carriers in his inoculated animals, and Nichols,¹⁶ in his earlier experiments, could not produce this condition with any degree of certainty. However, Doerr could get fairly high percentages of carriers, as could Gay and Claypole,¹⁷ and it would appear from later writings that Nichols himself was finally successful. In fact, it is now certain that if the proper technic is employed, the establishing of carriers is an easy matter. Various methods of injecting the culture have been and still are employed, some favoring direct injection into the gallbladder—(Hailer and Rimpau)¹⁸ and others injection into the mesenteric vein (Nichols). But ordinarily the marginal vein of the ear is used, as it proves quite satisfactory and is easy of access.

It was not long after the discovery of the gallbladder localization of typhoid bacilli, that attention was drawn to the danger that might result from the spread of infection either by healthy carriers or by convalescents, by means of stools and urine. Petruschy,¹⁹ in 1898, reported a case in which there was direct evidence that a healthy attendant had been infected by the urine of a convalescent. R. Koch,²⁰ however, was one of the first to emphasize the importance of the close observation of convalescents and carriers and advocated the establishment of research stations in those parts of the country in which typhoid fever was prevalent. It was his idea to carefully examine the stools and urine of all suspected carriers and convalescents and to safeguard other people as much as possible. The importance of detecting these carriers may be realized when one examines the reports of various investigators on the percentage of carriers that they have been able to detect. A chronological summary of the percentage of carriers reported by different authorities during approximately the last 10 years, is contained in a recent treatise on typhoid fever by Gay.²¹ Taking the average of these figures, we find that 4.3 per cent. gives a reasonable basis on which to judge our carrier percentage. That is to say, of every 100 cases of definitely proven fever, four or over harbor and eliminate typhoid bacilli for 3 months or more after recovery from the disease. Adding to these those persons who are carriers without any history of typhoid fever, it is very probable that the number is in reality greater than is normally estimated. It is this latter type, namely, the healthy "carrier" that is especially liable to be

¹⁴ Jour. Hyg., 1911, 7, p. 202.

¹⁵ Deutsch. med. Wchnschr., 1912, 38, p. 2397.

¹⁶ Jour. Exper. Med., 1916, 24, p. 497.

¹⁷ Arch. Int. Med., 1913, 12, p. 613.

¹⁸ Arbeit. a. d. Kais. Gesundh., 1912, 36, p. 409.

¹⁹ Centralbl. f. Bakteriol., 1898, 23, p. 577.

²⁰ Veröffentlichungen A. D. Militärsanitätswesen, 1092, 21.

²¹ Typhoid Fever Considered As a Problem of Scientific Medicine, 1918.

overlooked and consequently an important factor in the spread of infection through the handling of foodstuffs, especially milk and the like. The isolation of typhoid bacilli from the feces of such persons may not always be easy. Some carriers seem to eliminate the organisms almost continually, whereas with others, this elimination appears to be spasmodic. In certain suspected carriers where daily cultures have given negative results, purgatives such as elaterin, etc., are given to bring down material from the duodenum. However, animal experimentation has shown that gallbladder infection may still be active, although platings of intestinal contents are negative.

It is most natural that attempts to cure this condition should follow close in the wake of its discovery. One of the first put forth was that suggested by Forster,²² namely the administration of bile salts or dried bile. By this he hoped to increase the secretion of bile and thereby to wash out the organisms from the gallbladder. In 1910, Tsuzuki and Ishida²³ reported on some cases treated with iodine in the form of potassium iodide, in conjunction with Fowler's solution, with promising results, basing their opinion on the fact that the excreta of persons so treated cleared up more quickly than those of untreated cases. Conradi²⁴ used rectal injections of chloroform mixed with milk and cream and claimed very satisfactory results with laboratory animals. Knick and Pringsheim²⁵ found that the bile of dogs treated per os with large doses of menthol, methylene, hippuric acid and urotropin, was found to be bactericidal or strongly inhibitive to the growth of *B. typhosus* in broth. Mercurous chloride, sodium salicylate, salicylic acid, oil of turpentine and methylene blue had little or no effect. Uhlenhuth and Messerschmidt²⁶ tried monochloroacetylcholic acid, salicylate of copper, arsphenamin, phosphorus and colloidal mercury with negative results. Hailer and Rimpau²⁷ injected rectally methyl iodide, ethylbromide, chloroform, and iodoform in chloroform, all of these mixed with milk and cream before injection. They found the first two substances too toxic for use in laboratory animals, but reported occasional success with the use of iodoform in chloroform and also with bromoform, but do not recommend any of these for practical use. Kalberlah²⁸ gave his carriers (human) tincture of iodine in conjunction with charcoal and cites apparently cured cases, if one is justified in judging from repeated negative plates. Hailer and Wolf²⁹ tried phenols and ethereal oils, using xylol, thymol, and pyrogallol as representatives of the former and sandalwood oil, pinene, eucalyptol, and cinnamon oil, of the latter. It was only with the last named substance that they had any encouraging results. Nichols³⁰ in 1917 proposed an alkaline treatment of early gallbladder carriers. He suggests rendering the bile more alkaline by the administration of sodium bicarbonate, since bile with an increased alkalinity is destructive to typhoid bacilli. He was able to demonstrate an actual increase in the alkalinity of the bile after the administration of sodium bicarbonate and by such treatment was able to clear up several carriers. The treatment suggested by Hertz³⁰ may be mentioned here, namely, the injection of protein (milk) into the gluteal muscle. This causes a temperature rise, chill, and elimination of organisms and is claimed by the author to have been successful in several instances.

²² Verhandlung der Deutschen Patholog. Gesellschaft, 1907.

²³ Deutsch. med. Wchnschr., 1910, 36, p. 1005.

²⁴ Ztschr. f. Immunitätsforsch., 1910, 7, p. 158.

²⁵ Deutsch. Arch. klin. Med., 1911, 101, p. 137.

²⁶ Med. Klin., 1915, 11, p. 581.

²⁷ Arbeit. a. d. Kais. Gesundh., 1915, 48, p. 80.

²⁸ Jour. Am. Med. Assn., 1917, 68, p. 958.

³⁰ Wein. klin. Wchnschr., 1916, 29, p. 1290.

Soon after the value of prophylactic vaccination became established, vaccine therapy as a means of clearing up carriers was inaugurated and for a time considered successful. R. Koch³⁰ as early as 1902 suggested this vaccine treatment of the carrier. Irwin and Houston³¹ reported a typhoid carrier cured by the administration of an autogenous vaccine and Meader³² in 1910 likewise reported a case successfully treated. Following these many other apparent cures were reported and for a time this method of treatment was thought to promise a solution of the carrier problem. Johnston³³ reported some work on experimental rabbit carriers and claimed success in vaccine treatment of these. But certain of his results are so peculiar and divergent from those obtained by Doerr,¹ Bull,³⁴ Francke and Parker,³⁵ and others as well as in this laboratory, that one must question the validity of his results. For instance, he reports that in normal animals positive blood cultures appear in from 7-10 days after intravenous inoculation, and that the blood did not become sterile for from 30-60 days. In vaccinated animals the blood cultures were sterile in from 35-45 days after inoculation. In similar work on the same problem by the above authors, the blood cultures in normal animals became positive immediately on inoculation, and remained so constantly for from 10-14 days. Later than this it was rarely possible to get a positive blood culture.

Whatever may have been the apparent success of these earlier experimenters, repetition of this work and subsequent use of vaccine both in human and animal carriers have given little more than negative results. In fact, this method of treatment has been practically discarded and those who are seeking the solution of the carrier problem have been forced to turn again to the use of chemicals whose introduction into the body may be fatal to the organism but harmless to the host.

We have recently, in this laboratory, carried out a few experiments on the carrier rabbit using dyes which were known to be excreted through or broken down by the liver in hopes that their action on the organisms there localized might be powerful enough to cause their complete destruction. Safranin, methyl violet, crystal violet, ethyl violet, and Spiller's purple were the principal dyes used, but the results were entirely negative. Rabbits showed very little tolerance for most of these dyes and especially for methyl violet, indicating that these are unsuitable for this type of therapy and that further work along this line will have to be done with chemicals less toxic for experimental animals.

Knowing what we do about the typhoid bacillus, its entry into the body, localization in the various organs, and ultimate elimination from these organs, two points of interest suggest themselves to the investigator. First, is there any difference between the normal and immune animal, in the length of time that elapses before any or all organs become sterile after intravenous injection of living typhoid bacilli? And secondly, if there is any difference, just what is the mechanism involved and what factor or factors are responsible for this

³⁰ Lancet, 1909, Jan. 30, 1909, Vol. 115.

³² Bull. Johns Hopkins Hosp., 1910.

³³ Jour. Med. Research, 1912, 27, p. 177.

³⁴ Jour. Exper. Med., 1915, 22, p. 475; *ibid.*, 1916, 23, p. 419.

³⁵ Jour. Med. Research, 1919, 39, p. 301.

ability of the immune rabbit to so quickly rid itself of large numbers of living organisms? Typhoid, as we know, is a bacteremia, and therefore the bacilli must be carried to all parts of the body by the circulating blood. The question arises as to whether the organisms are killed off simultaneously in the blood and tissues, or remain in the organs after blood cultures are negative.

Doerr was the first to touch on this first phase of the mechanism of resistance and made cultures from the liver, spleen, blood and marrow of rabbits previously inoculated intravenously with living bacilli. In his experiments, carried out on normal animals alone, he found a rapid elimination of the organisms by all of the organs, and after the 14th day could not isolate them from the blood or any of the viscera, except the gallbladder. No attempts to determine the relative number of bacilli per given weight of organ were made, positive or negative results being based on the streaking of plates with the cut surfaces of organs or on the culturing of small pieces of organs in broth. Bull has also done considerable work on tracing the organisms shortly after intravenous injection, particularly as to their disappearance from the blood. He used only a small number of organisms ($\frac{1}{35}$ - $\frac{1}{40}$ agar slant) and his findings were those taken from 1 minute to one-half hour after injection and with normal rabbits alone. He found that the bacilli disappear very rapidly from the organs and especially from the circulating blood so that when $\frac{1}{40}$ of a culture of *B. typhosus* was injected, in 15 minutes only 1 organism per c c of blood could be isolated. Bartlett has worked on the intravenous inoculation of dogs with *Micrococcus aureus* and found that the organisms were rapidly taken up particularly by the liver and spleen, but that these same organs were the first to become sterile. Recently, the work of Parker and Francke, on the fate of intravenously injected typhoid bacilli into normal and immune rabbits, appeared. The technic used in estimating the number of bacteria per given weight of organ was similar to that used in experiments on the same problem described in this paper, with the exception that the number of bacteria injected in our experiments was probably smaller and the observations extended over a longer period of time. The authors mentioned found large numbers of bacilli in the organs of their animals and could demonstrate their rapid decrease, but concluded that there was little or no difference in the bactericidal properties of normal and immune rabbits. It would seem, however, that too many organisms were injected and

observations made over too short a period of time to fully warrant the conclusions drawn by them, conclusions which are not verified by the experiments described in this paper.

The second point which comes to mind is somewhat more difficult of approach. It is, namely, the mechanism involved in this ability of the body or body cells to rid themselves of the offending organisms. In other words, just what tissue or tissues are responsible for or take part in the restoring of the body to normal conditions. Is it the blood or tissue cells, or is it an interaction of these two that is responsible? Bull believes that the bacteria are clumped in the blood stream and accumulate in the capillaries of the organs especially the liver and spleen, where they are ingested by the polymorphonuclear leukocytes and destroyed. But this does not explain why the blood of immune rabbits is nonbactericidal even when large numbers of leukocytes are present or artificially introduced. Johnston claims that the bactericidal properties of normal serum for typhoid bacilli are practically nonexistent, whereas these properties are increased by vaccination. But the work of Bull, Teague, Buxton and others, and our own experiments would seem to prove the contrary to be true, namely, that normal serum is highly bactericidal to typhoid bacilli, whereas this power in highly immunized animals is practically nil.

THE FATE OF TYPHOID BACILLI IN NORMAL AND IMMUNE RABBITS

Normal Rabbits.—The question of the distribution and destruction of large numbers of living typhoid bacilli which have been injected intravenously into normal rabbits must be solved by determinations extending over weeks rather than minutes. In the following tabulated experiments care was taken that the number of organisms injected was as uniform as possible. A technic similar to that of Gay and Claypole, by which they were able to produce a high percentage of carriers, was followed.

Tubes of definite diameter (1.8 cm.) were used, containing 10 per cent. blood agar and slanted on an especially constructed tray, so that the slant extended almost to the cotton plug. The strain used was known as No. 3 and was isolated some years ago. Using a 24-hour culture grown in these standard tubes, Gay and Claypole estimated that each slant yielded 1,400,000 million organisms. One third of such a culture (approximately 460,000 million bacteria) was injected (with one or two exceptions, which are noted) into the marginal ear vein. The introduction of this large number of living bacteria proves fatal to a small fraction of the animals used in an experiment, within

48 hours after inoculation, but those surviving usually live for months, or indefinitely. Of the rabbits inoculated with living cultures, 92 per cent. became carriers. None of the animals dying from 4-24 hours after inoculation are included in Table 1, but all such animals were examined and bile cultures made which in practically all cases showed large numbers of typhoid bacilli. Of the animals killed, 86 per cent. were chronic carriers.

The technic used was as follows: the animal was killed by a blow at the back of the neck. After making a small preliminary incision with aseptic precautions, sufficient blood was taken from the heart for culture and subsequent tests for agglutinating titer. After this, the fur having been closely clipped or shaved, and the abdominal surface thoroughly wet down with alcohol, a midline incision was made. Small pieces of the various organs to be cultured were removed with sterile instruments and placed in petri dishes which had previously been weighed. The spleen, liver, kidney, lymph nodes and bone marrow were used. Culture's were also made from the gallbladder and urine when possible. Rectal and duodenal plates were occasionally made, on litmus lactose and Endo mediums. The Petri dishes containing these sections were then re-weighed to determine the exact amount of tissue, and these pieces were macerated in small sterile hand mortars. Into each mortar was poured sufficient broth that each one-tenth gm. of tissue would be suspended in 1 cc of fluid. These broth-tissue mixtures were poured into test tubes and allowed to stand at room temperature for half an hour, to allow the larger masses to settle and produce a more uniform suspension. At the end of this time 1 c.c. of the tissue extract was added to approximately 9 of melted agar and the whole plated. Colonies were counted after 24 hours at 37 C.

It will be noted, in the case of normal rabbits, that within one-half an hour typhoid bacilli are found in all organs with especially large numbers in the blood, liver, spleen and bone marrow. No attempts have been made to determine how soon the bacillus appeared in the various organs, as this has been clearly worked out by Bull. The number of organisms in the blood rapidly decreases during the first few hours, but their persistence here and in the other organs seems to last until about the 14th day after inoculation. As has been noted by all writers on the subject of experimental carriers the typhoid bacillus was found in the bile long after it had disappeared from all other parts of the body, with the occasional exception of the bone marrow. I have so far made no attempt to trace its persistence in the bile beyond the 86th day.

From table 1 it may be seen that somewhat under 2 weeks must elapse before the organs of the normal animal become sterile. This is, of course, with the exception of the gallbladder. The blood, apparently, may clear up a short time before the organs, but this discrepancy in time is so short that it would seem very probable that the immune bodies arising in the serum, tissue cells or both, sterilize the blood and organs simultaneously, but that it is quite a while before the factor or

factors necessary for this phenomenon are available in sufficient quantity to rid the body of the invading micro-organism. Of further interest is the fact that by the time agglutinins have reached a high titer, the animal is sterile.

TABLE 1
BACTERIAL COUNT FROM TISSUES OF NORMAL RABBITS INJECTED INTRAVENOUSLY WITH
LIVING TYPHOID BACILLI

No.	Weight in Gm.	Time	Aggluti- nating Titer	Blood 1 cc	Bile ½ cc	Spleen	Liver	Kid- ney	Lymph	Mar- row	Duod- enal	Rec- tal	Urine
I	—	½ hr.	—	688	160	Inn	Inn	—	192	Inn	—	—	—
II	—	3 hr.	—	408	1,696	1,068	3,200	—	1,800	528+	—	—	—
904	3,700	6 hr.	—	54	18	960	800	—	2	Inn	—	—	—
902	4,300	24 hr.	—	20	Inn	Inn	750	—	2	Inn	—	—	—
24	3,500	24 hr.	—	30	28	Inn	Inn	400+	Inn	400+	—	—	—
Y	2,800	3 da.	1:100	34	Inn	800	240	—	60	Inn	—	—	—
17	2,250	3 da.	—	32	Inn	Inn	Inn	Inn	Inn	Inn	—	Inn—	—
22	1,850	7 da.	1:10,000	16	Inn	60	Inn	8	Inn	Inn	—	—	24
845	2,400	10 da.	1:5,120	0	Inn	16	6	—	150	Inn	—	—	—
367	1,500	10 da.	—	—	Inn	—	—	—	—	—	—	—	—
19	2,600	14 da.	1:6,400	0	Inn	0	0	0	0	0	—	—	0
478	2,250	15 da.	1:800	0	Inn	0	0	0	0	0	0	0	0
99	2,100	16 da.	1:3,200	0	Inn	0	0	0	0	0	—	—	0
20	2,950	17 da.	1:1,600	0	0	0	0	0	0	0	0	0	0
226	1,800	18 da.	—	—	Inn	—	—	—	—	—	—	—	—
X	2,200	21 da.	1:162,000	0	Inn	0	0	0	0	Inn	—	—	—
147	1,900	21 da.	—	—	Inn	0	0	0	0	0	—	—	—
434	2,700	23 da.	1:800	0	0	0	0	0	0	0	0	0	0
437	2,550	23 da.	1:400	0	Inn	0	0	0	0	0	0	0	0
96	2,050	30 da.	1:1,600	0	0	0	0	0	0	0	0	0	0
205	1,900	35 da.	1:1,600	0	Inn	0	0	0	0	0	0	0	0
143	1,700	42 da.	1:400	0	Inn	0	0	0	0	—	Inn	Inn	0
152	—	86 da.	—	—	Inn	—	—	—	—	—	—	—	—

Colonies per plate inoculated with 1 cc tissue-extract.

— = not cultured.

Inn = innumerable.

0 = sterile plate.

Nos. 143, 147, 152 = given ¼ blood agar slant instead of ½.

Immune Rabbits.—We find entirely different results in the destruction of the bacilli in highly immune animals. Cultures killed by heat were used for this immunization, each agar slant being washed off with 8 c.c. salt solution, and this emulsion heated to 60 C. for 30 minutes. This suspension of bacteria was tested for sterility before using.

One-sixteenth of a culture (½ cc) was the initial amount injected, this being followed by 1 cc on each of the next 2 successive days. After a lapse of 4 days another series of injections was given, usually in amounts of 1, 1½ and 2 cc. This was usually found sufficient to produce a serum of high agglutinating titer for typhoid bacilli. Further injections were given to those animals that had not been used for 2 weeks or more after the last series of injections, and the agglutinating power of whose serums had dropped appreciably.

These immunized rabbits presented, after inoculation, an entirely different appearance from the normal injected with living culture. Whereas, shortly after inoculation with living bacteria, the latter appeared sick and drooping, and within a period of 24 hours would lose as high as 400 grams in weight, the immunized rabbits remained lively and lost very little weight, giving little or no evidence of any physical inconvenience caused by the injection of such large numbers of living typhoid bacilli.

TABLE 2
BACTERIAL COUNT FROM TISSUES OF IMMUNIZED RABBITS INJECTED INTRAVENOUSLY
WITH LIVING TYPHOID BACILLI

No.	Weight in Gm.	Time	Aggluti- nating Titer	Blood 1 c c	Bile ½ c c	Spleen	Liver	Kid- ney	Lymph	Mar- row	Duod- enal	Rec- tal	Urine
60	2,350	5 hr.	1:12,800	30	0	Inn	Inn	38	16	Inn	—	—	—
59	2,250	7 hr.	1:12,800	50	0	Inn	Inn	25	0	Inn	—	—	20
61	2,100	8 hr.	1:12,800	24	0	Inn	Inn	0	3	Inn	—	—	0
103	1,900	12 hr.	1:12,800	30	0	Inn	Inn	21	7	800	—	—	0
100	2,100	18 hr.	1:3,200	400	Inn	Inn	400	Inn	0	Inn	—	—	Inn
468	1,950	18 hr.	1:3,200	160	Inn	Inn	Inn	0	0	Inn	—	—	10
58	2,400	18 hr.	1:12,800	0	0	0	0	0	0	0	—	—	0
150	2,450	24 hr.	1:16,000	0	0	0	0	0	0	0	0	0	0
172	2,200	24 hr.	1:6,400	0	0	0	0	0	0	14	—	—	—
153	2,600	24 hr.	1:3,200	0	0	0	0	0	0	—	—	—	—
186	2,700	28 hr.	1:6,400	0	0	0	0	0	0	0	—	—	—

A comparison of table 2 with table 1 shows a remarkable difference between the normal and immune rabbits in their ability to rid themselves of the organisms injected. Whereas, in the normal rabbit, the typhoid bacilli can be isolated from the organs for at least 10 days after injection, immune animals are, but with occasional exceptions, sterile in less than 24 hours. An ability to quickly destroy large numbers of bacteria has been developed in the immune animals, a power to destroy in 24 hours or less the same number of organisms that it takes the normal rabbit almost 2 weeks to dispose of. Since it is evident that the rabbit carrier is analogous to the human, in general localization of the bacteria in the organs especially in the gallbladder, and their elimination with the feces, there is the possibility that the protection afforded human beings by vaccination may be compared with this resistance of immunized animals against living organisms. At any rate a study of this rapid destruction of typhoid bacilli by the immune rabbit may at least serve as an approach to an understanding of the mechanism of the immunity conferred in man by vaccination or recovery from disease.

EXPERIMENTS ON THE MECHANISM OF RESISTANCE

With this analogy in mind, a study of the mechanism of this immunity reaction was undertaken to determine, if possible, what factor or factors in the animal body are concerned in the development of the new property which makes its appearance after immunization.

Some years ago a set of experiments was carried out in this laboratory to test the protective value—in vivo—of typhoid immune rabbit serum. The M. L. D. of a certain laboratory strain (No. 3) of typhoid bacilli was determined by repeated intraperitoneal injection of varying dilutions of the culture

in mice. This having been determined, the protective value of the immune serum was tested, by injecting it into mice 6 hours before the injection of culture. Using a set amount of culture, various amounts of serum were tested, and it was found that as small a dose of serum as 0.01 cc protected mice against 2-3 M. L. D. of bacteria. Even when the injections of serum and bacilli were made simultaneously there was a certain amount of protection, although not as marked as when the serum was given 6 hours previous to the administration of the culture. Serum injected a short time after the culture was of no value, nor could any protecting power of normal serum be demonstrated no matter when injected. This would seem to indicate that new properties acquired by the serum are responsible for the protection of immune animals but subsequent experiments have shown that although the bacteriolytic power of the serum in vivo is very great, in vitro it has no effect on the organisms.

A repetition of the work by Bull, Buxton, Teague and Williams, and others on the comparative bactericidal properties of normal and immune serums in vitro, was carried out, with the results similar to those obtained by them. It was found that 1/10 cc or less of normal serum was usually sufficient to kill 1/10 cc of a 1:10,000 dilution of a 24-hour broth culture of *B. typhosus*. Repeated platings showed this to be about 1¼ million organisms. Immune serum, on the other hand, regardless of the agglutinating titer was nonbactericidal in amounts as large as 1 cc or more for the same number of organisms (Tables 3, 4).

TABLE 3
COMPARISON OF BACTERICIDAL POWER OF NORMAL AND IMMUNE SERUMS

				Subculture
1.	Fresh normal serum	0.5 cc +	0.1 cc 1:10,000 dilution of culture	0
2.	Fresh normal serum	0.4 cc +	0.1 cc 1:10,000 dilution of culture	0
3.	Fresh normal serum	0.3 cc +	0.1 cc 1:10,000 dilution of culture	0
4.	Fresh normal serum	0.2 cc +	0.1 cc 1:10,000 dilution of culture	0
5.	Fresh normal serum	0.1 cc +	0.1 cc 1:10,000 dilution of culture	0
6.	Fresh normal serum	0.05 cc +	0.1 cc 1:10,000 dilution of culture	+
7.	Fresh immune serum (153)	0.5 cc +	0.1 cc 1:10,000 dilution of culture	+
8.	Fresh immune serum	0.4 cc +	0.1 cc 1:10,000 dilution of culture	+
9.	Fresh immune serum	0.3 cc +	0.1 cc 1:10,000 dilution of culture	+
10.	Fresh immune serum	0.2 cc +	0.1 cc 1:10,000 dilution of culture	+
11.	Fresh immune serum	0.1 cc +	0.1 cc 1:10,000 dilution of culture	+
12.	Fresh immune serum	0.05 cc +	0.1 cc 1:10,000 dilution of culture	+

Agglutinating titer of No. 153 = 1:51,000. Subcultures made after 18 hours' incubation 0.1 cc of 1:10,000 dilution of culture = approximately 1¼ million organisms.

TABLE 4
CHANGE IN BACTERICIDAL POWER OF SERUM AFTER IMMUNIZATION

				Subculture
1.	No. 386 (before immunization)	0.5 cc +	0.1 cc 1:10,000 dilution of culture	0
2.	No. 386 (before immunization)	0.4 cc +	0.1 cc 1:10,000 dilution of culture	0
3.	No. 386 (before immunization)	0.3 cc +	0.1 cc 1:10,000 dilution of culture	0
4.	No. 386 (before immunization)	0.2 cc +	0.1 cc 1:10,000 dilution of culture	0
5.	No. 386 (before immunization)	0.1 cc +	0.1 cc 1:10,000 dilution of culture	0
6.	No. 386 (before immunization)	0.05 cc +	0.1 cc 1:10,000 dilution of culture	0
7.	No. 386 (after immunization)	0.5 cc +	0.1 cc 1:100,000 dilution of culture	+
8.	No. 386 (after immunization)	0.4 cc +	0.1 cc 1:100,000 dilution of culture	+
9.	No. 386 (after immunization)	0.3 cc +	0.1 cc 1:100,000 dilution of culture	+
10.	No. 386 (after immunization)	0.2 cc +	0.1 cc 1:100,000 dilution of culture	+
11.	No. 386 (after immunization)	0.1 cc +	0.1 cc 1:100,000 dilution of culture	+
12.	No. 386 (after immunization)	0.05 cc +	0.1 cc 1:100,000 dilution of culture	+

Attempts to reactivate the immune serum were carried out as follows: Immune serum was heated to 56 C. for one-half hour. It was then reactivated by the addition of small amounts of fresh normal serum. If the amount of normal serum used in the reactivation was that known to be insufficient in itself to kill the standard number of bacilli used, there was no inhibition of growth. If, however, more than this amount of normal serum was used, no growth occurred, showing that the normal serum acted independently and was equally potent whether in immune serum or saline, and that any bactericidal properties present were due to the normal serum alone (Table 5).

TABLE 5
REACTIVATION OF IMMUNE SERUM

			Subculture
1. Serum No. 153	0.5 cc + 0.1 cc culture (dilution 1:100,000).....		+
2. Serum No. 153	0.3 cc + 0.1 cc culture (dilution 1:100,000).....		+
3. Serum No. 153	0.1 cc + 0.1 cc culture (dilution 1:100,000).....		+
4. Serum No. 153 (56°)	0.4 cc + 0.1 cc culture (dilution 1:100,000) + 0.1 cc normal serum	0	
5. Serum No. 153	0.3 cc + 0.1 cc culture (dilution 1:100,000) + 0.1 cc normal serum	0	
6. Serum No. 153	0.2 cc + 0.1 cc culture (dilution 1:100,000) + 0.1 cc normal serum	0	
7. Serum No. 153	0.4 cc + 0.1 cc culture (dilution 1:100,000) + 0.05 cc normal serum	+	
8. Serum No. 153	0.3 cc + 0.1 cc culture (dilution 1:100,000) + 0.05 cc normal serum	+	
9. Serum No. 153	0.2 cc + 0.1 cc culture (dilution 1:100,000) + 0.05 cc normal serum	+	
10. Normal serum	0.3 cc + 0.1 cc culture (dilution 1:100,000).....	0	
11. Normal serum	0.2 cc + 0.1 cc culture (dilution 1:100,000).....	0	
12. Normal serum	0.1 cc + 0.1 cc culture (dilution 1:100,000).....	0	
13. Normal serum	0.05 cc + 0.1 cc culture (dilution 1:100,000).....	+	

Tests were also made, using high dilutions of immune serum, the possibility being suggested that the agglutination of the bacteria protected them from the bactericidal action of the serum. However, no destruction of the bacteria could be found in dilutions as high as 1 to 1 million, using sera whose agglutinating titer were 1/6,400 or above (Table 6).

To test for possible leukocytic properties, whose addition might be necessary for the action of immune serum on bacteria, the following experiments were carried out. Intrapleural injections of 8 cc of broth were given to rabbits late in the afternoon. Early the next morning 6-8 cc more broth were injected into each pleural cavity and the rabbits killed by exsanguination 2 hours later. The admixture of sterile sand to the broth to increase by irritation the leukocytic output, was found of no particular advantage. Eight to 10 cc of creamery fluid could be recovered from each pleural cavity, which was found to contain enormous numbers of leukocytes, mainly polymorphonuclears. This was collected in 2-3 cc of 1 per cent. sodium citrate, to prevent clotting, and the cells sedimented by gentle trifugalization. The supernatant fluid was then poured off and the suspension restored to its original volume with normal salt solution. Smears showed that these leukocytes had not suffered by this treatment.

This leukocytic suspension was then added to different dilutions of immune serum and living typhoid bacilli, to demonstrate, if possible, any bacteriolytic effect of the serum-leukocyte combination. But no inhibition of the bacterial growth could be demonstrated, nor have the leukocytes alone any lytic effect on the bacteria. Slides made at 20 and 40 minutes after the addition of the bacteria to the leukocytes and serum showed no phagocytosis. However, this would probably have been difficult to demonstrate because of the small number of bacteria introduced (Table 6).

TABLE 6
IMMUNE SERUM AND LEUKOCYTES

Immune Serum (fresh)—				Subculture
1.	No. 386	0.3 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
2.	No. 386	0.2 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
3.	No. 386	0.1 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
4.	No. 386	0.01 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
5.	No. 386	0.001 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
6.	No. 386	0.0001 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
7.	No. 386	0.00001 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
8.	No. 315	0.3 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
9.	No. 315	0.2 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
10.	No. 315	0.1 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
11.	No. 315	0.01 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
12.	No. 315	0.001 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
13.	No. 315	0.0001 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
14.	No. 315	0.00001 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:10,000)	+
15.	No. 386	0.3 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:100,000)	+
16.	No. 386	0.3 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:100,000,000)	+
17.	No. 315	0.3 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:100,000)	+
18.	No. 315	0.3 cc	+ leukocytes 0.2 cc + 0.1 cc culture (1:100,000,000)	+
Normal Serum (fresh)—				
19.	0.5 cc	+ 0.1 cc culture (1:10,000)	0
20.	0.4 cc	+ 0.1 cc culture (1:10,000)	0
21.	0.3 cc	+ 0.1 cc culture (1:10,000)	0
22.	0.2 cc	+ 0.1 cc culture (1:10,000)	0
23.	0.1 cc	+ 0.1 cc culture (1:10,000)	0
24.	0.05 cc	+ 0.1 cc culture (1:10,000)	+
Immune Serum (fresh)—				
25.	No. 386	0.4 cc	+ 0.1 cc culture (1:10,000)	+
26.	No. 386	0.3 cc	+ 0.1 cc culture (1:10,000)	+
27.	No. 386	0.2 cc	+ 0.1 cc culture (1:10,000)	+
28.	No. 386	0.1 cc	+ 0.1 cc culture (1:10,000)	+
29.	No. 386	0.05 cc	+ 0.1 cc culture (1:10,000)	+
30.	No. 386	0.01 cc	+ 0.1 cc culture (1:10,000)	+
31.	No. 386	0.001 cc	+ 0.1 cc culture (1:10,000)	+
32.	No. 386	0.0001 cc	+ 0.1 cc culture (1:10,000)	+
33.	No. 386	0.00001 cc	+ 0.1 cc culture (1:10,000)	+
34.	No. 315	0.4 cc	+ 0.1 cc culture (1:10,000)	+
35.	No. 315	0.3 cc	+ 0.1 cc culture (1:10,000)	+
36.	No. 315	0.2 cc	+ 0.1 cc culture (1:10,000)	+
37.	No. 315	0.1 cc	+ 0.1 cc culture (1:10,000)	+
38.	No. 315	0.05 cc	+ 0.1 cc culture (1:10,000)	+
39.	No. 315	0.01 cc	+ 0.1 cc culture (1:10,000)	+
40.	No. 315	0.001 cc	+ 0.1 cc culture (1:10,000)	+
41.	No. 315	0.0001 cc	+ 0.1 cc culture (1:10,000)	+
42.	No. 315	0.00001 cc	+ 0.1 cc culture (1:10,000)	+
43.	No. 369	0.4 cc	+ 0.1 cc culture (1:10,000)	+
44.	No. 369	0.3 cc	+ 0.1 cc culture (1:10,000)	+
45.	No. 369	0.2 cc	+ 0.1 cc culture (1:10,000)	+
46.	No. 369	0.1 cc	+ 0.1 cc culture (1:10,000)	+
47.	No. 369	0.05 cc	+ 0.1 cc culture (1:10,000)	+
48.	No. 369	0.01 cc	+ 0.1 cc culture (1:10,000)	+
49.	No. 369	0.001 cc	+ 0.1 cc culture (1:10,000)	+
50.	No. 369	0.0001 cc	+ 0.1 cc culture (1:10,000)	+
51.	Leukocytes 0.2 cc	+ 0.1 cc culture (1:10,000)	+

To test for the possible activity of the tissue cells in the rapid destruction of typhoid bacilli in the immune animal, the tissues of such animals were tested with respect to their destruction of the bacteria brought in contact with them. Highly immune rabbits were exsanguinated by bleeding from the carotid. Pieces of organs were coarsely ground in mortars with the aid of a small amount of sand, and to these were added a comparatively small number of typhoid bacilli (1/10 cc of a 1:500,000 dilution of broth culture). After incubation for 18 hours at 37 C. plates were streaked. All gave a good growth of typhoid bacilli. Attempts were also made to bring about bacteriolysis by the addition of homologous immune serum to these tissue-bacteria mixtures, but no inhibition of multiplication of the bacilli could be demonstrated (Table 7).

TABLE 7
IMMUNE SERUM AND TISSUES

	Culture
1. Liver (cut surface) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
2. Spleen (cut surface) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
3. Kidney (cut surface) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
4. Lymph node (cut surface) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
5. Bone marrow (cut surface) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
6. Liver (macerated) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
7. Spleen (macerated) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
8. Kidney (macerated) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
9. Lymph node (macerated) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
10. Bone marrow (macerated) + 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+
11. Liver (cut surface) + 0.1 1:500,000 dil. culture.....	+
12. Spleen (cut surface) + 0.1 1:500,000 dil. culture.....	+
13. Kidney (cut surface) + 0.1 1:500,000 dil. culture.....	+
14. Lymph node (cut surface) + 0.1 1:500,000 dil. culture.....	+
15. Bone marrow (cut surface) + 0.1 1:500,000 dil. culture.....	+
16. Liver (macerated) + 0.1 1:500,000 dil. culture.....	+
17. Spleen (macerated) + 0.1 1:500,000 dil. culture.....	+
18. Kidney (macerated) + 0.1 1:500,000 dil. culture.....	+
19. Lymph node (macerated) + 0.1 1:500,000 dil. culture.....	+
20. Bone marrow (macerated) + 0.1 1:500,000 dil. culture.....	+
21. 0.2 cc immune serum + 0.1 1:500,000 dil. culture.....	+

Unmacerated cut sections of organs were also inoculated to test for any possible action of the tissue cells that might have been destroyed by grinding. But there was no bacteriolysis, either when bacteria alone were used, or when immune serum was added. In fact, all these experiments showed as rapid a growth of the organism as there would be on any favorable culture medium.

It seems that we are confronted with some rather paradoxical facts concerning the mechanism of bacteriolysis in the body of the immune animal, namely:

1. Typhoid bacilli disappear more quickly from the organs of immune animals than from normal animals.

2. Macerated organs, from immune animals, cut sections, or their extracts are not bactericidal even on the addition of fresh immune serum.

3. Typhoid immune serum is nonbactericidal for typhoid bacilli in vitro.

4. Fresh normal serum is highly bactericidal for typhoid bacilli in vitro.

5. Fresh immune serum in vivo, has apparently a high bactericidal power.

6. Fresh normal serum in vivo has no protective power.

This would seem to indicate that the destruction of typhoid bacilli in the immune animal is due either to some interaction between the tissue cells and plasma in vivo, or to some other factor which has thus far been overlooked. The theory proposed by Teague, namely, that immunization causes a rapid filtering out of the immune bodies in the capillaries into the tissues, seems plausible were it not for the fact that the organs of highly immune animals exert no bactericidal action on living typhoid bacilli. Further work on this subject may lead to more definite conclusions than can here be given.

EXPERIMENTAL STREPTOCOCCIC TONSILLITIS

THE APPARENT INEFFICACY OF STREPTOCOCCIC VACCINE AS PROPHYLACTIC

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It has long been recognized that acute tonsillitis is of bacterial origin and infectious nature. Even as early as 1895, Fraenkel and Macintyre¹ called attention to this fact and, in the same year, Sendziak² noted the frequency of streptococci, staphylococci and corynebacteria in tonsillar cultures, taken in situ. Davis,³ in a series of cultures from the extirpated tonsils of 113 cases, found virulent hemolytic streptococci to be, as a rule, the predominant organism and observed that pneumococci were encountered more frequently on the surface of the tonsil, whereas streptococci occurred more commonly in the lacunae. The same author, in another work,⁴ described the actinomyces-like granules in the crypts of the tonsils and mentioned the occurrence of nonpathogenic, anaerobic, fusiform bacilli, streptococci and spirilla in them. More recently, Pilot and Davis⁵ isolated both hemolytic streptococcus and *S. viridans* from these granules. The green strains showed marked anaerobic tendencies, while the hemolytic did not. According to the classification of Holman,⁶ the hemolytic streptococci corresponded to *S. pyogenes* and *S. anginosus* and the viridans to *S. mitis* and *S. salivarius*. Holman encountered these four varieties most frequently in the nose and throat. The weight of evidence, both clinical and cultural, points to the causative organisms of acute tonsillitis as being streptococci or, in the minority of cases, staphylococci and the mode of dissemination to be either direct or nearly direct contact. Other factors, of course, such as age, climatic conditions and resistance-lowering processes may play a predisposing rôle.

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¹ Brit. Med. Jour., 1895, 2, p. 1018.

² Jour. Laryngol. (London), 1895, 9, p. 263.

³ Jour. Infect. Dis., 1912, 10, p. 148.

⁴ Ibid., 1914, 14, p. 144.

⁵ Ibid., 1918, 23, p. 565.

⁶ Jour. Med. Research, 1916, 34, p. 377.

Inoculations of living organisms into the nasopharynx have been made on the human subject by several observers. Usually viable cultures of *Staphylococcus aureus* were sprayed into the nares and throats of *B. diphtheriae* carriers. Page⁷ treated seven cases in this way with no untoward results. Catlin, Scott and Day⁸ encountered no cases of tonsillitis in eight instances. Lorenz and Ravenel⁹ reported one very mild case of laryngitis following the instillation of staphylococci in a series of seventeen cases, while Alden,¹⁰ in sixteen cases, noted "sore throat" in two and pharyngitis in one. Similarly, Davis¹¹ recounts the development of tonsillitis 26 hours after the introduction of staphylococci into the nasopharynx of a carrier of *B. diphtheriae*. This extremely low percentage of tonsillitis cases following such procedure is very interesting and may be attributed, in part, to the attenuation of the cultures employed.

I have not found any account of experimental throat infections in man due to the use of secretions from the upper respiratory passages of the sick.

The present report deals with the occurrence of sixteen cases of tonsillitis, due to infection with secretions from the sick, which developed during the course of some experiments on human volunteers, conducted by medical officers of the U. S. Navy and U. S. Public Health Service, in an attempt to ascertain, if possible, the mode of dissemination and cause of influenza.

These experiments were three in number. The first two took place simultaneously at San Francisco and Boston during November and December, 1918, the third at Boston during February and March, 1919.

A detailed account of these researches will be published in a forthcoming bulletin of the Hygienic Laboratory, U. S. Public Health Service. It is not the purpose of this communication to discuss the results of these investigations from the standpoint of influenza, but to emphasize the production of tonsillitis in 16 of the volunteers. For this reason, only the methods and data pertinent to the subject at hand will be indicated.

All of the 155 volunteers were from the enlisted personnel of the U. S. Navy. Their ages ranged from 15 to 36 years, the average being, approximately, 20 years. Save for the occasional incidence of hypertrophic tonsils, the men were

⁷ Arch. Int. Med., 1911, 7, p. 16.

⁸ Jour. Am. Med. Assn., 1911, 57, p. 1452.

⁹ Jour. Am. Med. Assn., 1912, 59, p. 692.

¹⁰ Jour. Am. Med. Assn., 1913, 60, p. 1876.

¹¹ Jour. Am. Med. Assn., 1913, 61, p. 393.

in excellent condition. The histories revealed the fact that they had enjoyed good health, some never having been ill. Less than 10% of them had experienced repeated attacks of tonsillitis and none had suffered from "sore throat" for several weeks before the experiments began.

The essential differences between the three groups were that the one at San Francisco had been under absolute quarantine since Sept. 23, 1918, while the Boston contingents had experienced a contact with influenza patients which varied from practically no exposure to the disease during the recent pandemic to the most intimate association with it. There were no cases of influenza on the San Francisco station until Dec. 6, 1918, sixteen days after the quarantine had been removed. For purposes of experimentation, therefore, the San Francisco group would have been ideal were it not for the fact that, on Oct. 12, 15, and 18, 1918—approximately a month before the experiments were inaugurated—the entire personnel had been given a vaccine of which one mil contained:

B. influenzae.....	5 billion
Pneumococcus (type I).....	3 billion
Pneumococcus (type II).....	3 billion
Pneumococcus (type III).....	1 billion
Streptococcus hemolyticus.....	100 million

The doses were 0.5, 0.8 and 1.0 mil, respectively. The inoculation produced a certain degree of local reaction along with headache, generalized pains and a slight increase in temperature. These were most marked after the second infection, but never persisted longer than 48 hours. An account of this procedure has been reported by Minaker and Irvine.¹²

SUMMARY OF DATA OBTAINED IN THE STUDY OF EXPERIMENTAL STREPTOCOCCIC TONSILLITIS

Place	Time	Total Number of Volunteers	Total Number of Inoculations	Total Number Inoculations of Crude Secretions into Nasopharynx	Total Number Volunteers Receiving Crude Secretions into Nasopharynx	Total Number Cases of Tonsillitis	Percentage of Volunteers Inoculated with Crude Secretions Who Developed Tonsillitis
San Francisco	Nov.-Dec., 1918	50	40	14	14	3	21.4
Boston	Nov.-Dec., 1918	62	104	49	32	1	2.0
Boston	Feb.-Mar., 1919	43	82	61	40	12	19.6
Total.....	155	226	124	86	16	12.9

From the table it can be seen that 155 men were utilized in the three experiments. On these men 226 inoculations were made, of which 124 consisted in the transference of crude nasopharyngeal washings and bronchial secretions from certain individuals presumably ill of influenza into the noses and throats of 86 different men. Sixteen of these men developed tonsillitis. Only two of them showed hypertrophic tonsils before inoculation. No volunteer receiving filtered secretions showed a subsequent throat condition.

¹² Jour. Am. Med. Assn., 1919, 72, p. 847.

In the first Boston experiments tonsillitis occurred in only one of the forty-nine (2%) volunteers receiving crude secretions. In the second series of experiments at Boston, 12 of sixty-one (19.6%) became ill of this disease. Six of these 12 cases occurred in one squad where, in an endeavor to obtain an extremely early case of influenza, nasopharyngeal washings and bronchial secretions were procured from an individual who was suspected to be developing influenza, but who later proved to have tonsillitis. If these were disregarded, it would leave 6 cases in 61 inoculations, or 9.8% for this group. In the San Francisco experiments, tonsillitis occurred in 3 of 14 men (21.4%) into whose noses were instilled unfiltered nasopharyngeal washings and bronchial secretions.

The cases of tonsillitis referred to in this report were clinically typical. All exhibited more or less purulent exudate in the crypts of one or both tonsils at some stage of their illness. They all made an uneventful recovery, except one man who developed a right-sided otitis media which proceeded to recovery.

The incubation period was, in all but two instances, between 36 and 72 hours. The two exceptions were encountered in the second Boston investigations, and the periods of incubation were 5 and 6 days, respectively. The cases in a given group became ill within a few hours of each other, so that, even though the other members of the group were undergoing experimentation they were in contact with those developing the disease, the time element clearly indicated a common source of infection.

In every case a hemolytic streptococcus was found to be the predominating organism in the cultures taken from the tonsils in the early stages of illness, and it was noted that on the fresh, human blood-agar plate the colonies were very similar to those seen in the cultures from the donors of the respective groups. All corresponded to the "beta" type of Smith and Brown,¹³ and Smillie.¹⁴ No difficulty was occasioned in cultivating them, aerobically, on human blood agar (5%) and serum broth. Those cultures from donors and recipients which were transplanted to carbohydrate mediums fermented lactose and salicin with the formation of acid, corresponding to *S. pyogenes* in Holman's classification.

¹³ Jour. Med. Research, 1914-15, 31, p. 455.

¹⁴ Jour. Infect. Dis., 1917, 20, p. 45.

By reverting to the table, it will be noted that the highest percentage of cases of tonsillitis occurred in the San Francisco experiments. As mentioned before, the volunteers in this group had received, one month previously, a vaccine, 1 mil of which contained, along with other organisms, 100 million hemolytic streptococci which had been isolated from the upper respiratory passages of two influenzal patients. In neither of the Boston series of experiments were any volunteers used who had been given vaccine. It is not intended that one deduce from this that the vaccine sensitized the recipients to subsequent streptococcal infection.

Naturally, in this regard, several phases of the problems of vaccino-therapy present themselves. The early endeavors of Pasteur and Koch have been amplified and given to the profession in a more concrete form by Wright.¹⁵ In the last decade of the 19th century von Behring and Knorr¹⁶ found that rabbits immunized by a given strain of streptococci were protected against that strain. Since this time many investigations have been pursued to ascertain the efficacy of active and passive immunization against streptococci, both as prophylactic and therapeutic measures. As a rule, the experimental work has been carried out on rabbits, an animal known to be susceptible to this organism. Weaver¹⁷ concluded that killed streptococci, injected into an animal, may raise its resistance to the living strain. Simonds,¹⁸ as well as Tunnicliff¹⁹ have called attention to the effect of streptococci on the streptococco-opsonic index in scarlet fever—causing a positive phase after a primary negative phase. Moore²⁰ noted that the injection of a streptococcal vaccine protected many rabbits from arthritis when hemolytic strains of streptococci were used, the immunity lasting 40 days. Clinically, the use of streptococcal vaccines can be summed up by the statement of Gay,²¹ who, in a comprehensive review of the streptococcus problem, says that “streptococcus vaccines have not been used to any considerable extent as a means of protection against streptococcus infection.”

The early observers in the field of vaccino-therapy were insistent that vaccine be autogenous when employed as a curative agent. This,

¹⁵ *Lancet*, 1902, 1, p. 651; 1907, 2, p. 493.

¹⁶ *Centralbl. f. Bakteriologie*, 1892, 12, p. 192.

¹⁷ *Tr. Assn. Am. Phys.*, 1910, 8, p. 223.

¹⁸ *Jour. Infect. Dis.*, 1907, 4, p. 595.

¹⁹ *Jour. Infect. Dis.*, 1907, 4, p. 304.

²⁰ *Jour. Infect. Dis.*, 1914, 15, p. 215.

²¹ *Jour. Lab. and Clin. Med.*, 1918, 3, p. 722.

of course, is impossible when the vaccine is used as a prophylactic, but Rosenow²² advocates utilization of organisms found in the upper respiratory passages of persons in a given community during a given outbreak. Mellon²³ thought that the best results were obtained from the use of streptococci of proved immunizing power.

The organisms employed in this vaccine were killed by heating to 56 C. for 1 hour and then mixing with 0.5% phenolized salt solution. As Wayson²⁴ indicates, the guiding principle for vaccines has been conservation of all the "immunogenic qualities" and the destruction of infectivity of the principles of the vaccine. There is some reason to believe that these "immunogenic qualities" are partially impaired by heating to 56 C. for an hour, as Weaver¹⁷ reports better results with streptococci killed in strong galactose solutions at 35 C. and states that heating interferes with the antigenic properties of streptococci. However, the heat killed method, plus a weak solution of phenol or tricresol, has proven efficient in the hands of numerous investigators and is the procedure generally chosen.

The advocates of vaccinothrapy usually recommend beginning with small doses rather than large ones. Wright, himself, laid stress on the fact that the dosage should be sufficiently small to excite only a mild, local and constitutional reaction. Adami,²⁵ in discussing the basic factors governing the use of vaccines, emphasized this point. Simonds¹⁸ showed, in his experimental work on rabbits, that relatively small doses of streptococci may cause a greater rise in the opsonic index than large doses. On the other hand, Cecil and Austin,²⁶ in their work at Camp Upton, with pneumococcal vaccine, observed that the degree of response to the vaccination appeared to be dependent on the total dosage of each type of pneumococci administered. Boughton²⁷ reports good results by using an initial dose of 100 million homologous, galactose-killed streptococci in local streptococcic complications of scarlatina and erysipelas. Stitt²⁸ quotes Wilson as recommending from 6-68 million as the minimum and maximum doses for streptococcic vaccines. Kolmer²⁹ gives from 25-200 million per mil,

²² Jour. Am. Med. Assn., 1919, 72, p. 31.

²³ Med. Record, 1915, 87, p. 809.

²⁴ Jour. Am. Med. Assn., 1917, 67, p. 267.

²⁵ Tr. Assn. Am. Phys., 1910, p. 211.

²⁶ Jour. Exper. Med., 1918, 28, p. 19.

²⁷ Jour. Infect. Dis., 1910, 7, p. 99.

²⁸ Practical Bacteriology, Blood Work, Parasitology, 1918, p. 214.

²⁹ Infection. Immunity and Specific Therapy, 1917, p. 223.

as the usual limits of dosage for a streptococcic vaccine. Thomas and Ivy³⁰ advocate from 2-200 million, with an average dose of 25-50 million, while Park and Williams³¹ vary the dose from 5-10 to 100 million or higher. Ely, Lloyd, Hitchcock and Nickson³² report beneficial results with respect to the prophylaxis of influenza in a large group of men when 0.25, 0.5 and 1 mil of a streptococcic vaccine, containing 250 million of these organisms—per mil—were given.

When it is remembered that the vaccine used on the volunteers contained approximately 100 million per mil and that a total of about 230 million were given, it would appear that the amount was well within logical limits, particularly in the light of the relatively mild focal or constitutional reactions among those who received it.

From what has been said, it is reasonable to suppose that in the choice of organisms, the mode of preparation, the dosage and the manner of administration, there was nothing of a technical nature which would preclude the vaccine from being efficacious.

SUMMARY

During the recent investigations of 155 human volunteers to study the epidemiology of influenza, 16 different individuals developed tonsillitis apparently due to hemolytic streptococci.

All cases of tonsillitis occurred in men receiving crude nasopharyngeal washings and bronchial secretions from early, acute, typical, uncomplicated influenza patients—with exception of six cases in which the common donor had acute tonsillitis; no cases of tonsillitis occurred in those into whose nares were instilled filtered nasopharyngeal washings and bronchial secretions from influenza donors.

The causative organisms of the tonsillitis were very similar to the hemolytic streptococci isolated from the nasopharyngeal washings and bronchial secretions of the donors of the respective groups.

Three of the cases of tonsillitis developed in persons who had received, one month previously, a vaccine containing, in addition to other organisms, three presumably adequate doses of hemolytic streptococci.

³⁰ Applied Immunology, 1915, p. 251.

³¹ Pathogenic Micro-organisms, 1917, p. 590.

³² Jour. Am. Med. Assn., 1919, 72, p. 24.

TWO INSTRUCTIVE OUTBREAKS OF TYPHOID FEVER

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There are a variety of channels through which the contagion of typhoid fever may be disseminated in such a manner as to cause an epidemic, but foremost among these are public water and milk supplies. In past years it has sometimes been difficult to determine positively which of these two routes was the guilty one in a given case, but with the development of modern epidemiologic methods this difficulty has been very largely overcome. Too many physicians and local health officers, however, still persist in making a guess at what might be the source of infection and then allow the matter to rest, or else collect samples of drinking water and milk at random and send them to a bacteriological laboratory to be examined for typhoid bacilli. Experience has demonstrated that these guesses are about as likely to be wrong as right, and that the task of finding the source of infection is most likely to be successful if we start with the patients and from them ascertain as nearly as possible when, where and under what conditions each case has occurred. This is clearly illustrated by one of the outbreaks of typhoid fever that I was called on to investigate recently in McGill, Nevada.

McGill is a small mining town of approximately 3,000 inhabitants. It is supported entirely by labor in copper smelters and related industries, and the entire village is owned by the Nevada Consolidated Copper Company. It has two separate public water supplies, each of which supplies a definite section of the village. One of these sources of supply is from a shallow well (known locally as "Domestic Spring" supply) located in the northern part of the village and the other is from a small mountain stream ("Duck Creek") which is carried out of the mountains in ditches and is then piped into the village. The latter water is passed through a pressure filter to remove suspended matter, but the filter does not remove bacteria from the water. The greater part of the village is sewered, but only a relatively small percentage of the toilets have sewer connection. Most of the outside privies are in a neglected and insanitary condition. Many of them are not provided with a door but are protected from view by a board screen placed about 3 feet in front of the entrance. The seat is nothing more than a rail, and the pit is not covered in any way.

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August 24, 1918, there was received at this laboratory a telegram from McGill stating that samples of milk and water were being shipped by express to determine whether or not they contained typhoid bacilli. The samples were received in good order on the following day, packed in ice, and the water samples (three in number) were submitted to the regular bacteriologic analysis. The results showed that none of the samples contained *B. coli* in 1 c.c. quantities, but sample 1 (dairy well), and sample 2 ("Duck Creek" supply), yielded *B. coli* in 5 c.c. quantities, and the total number of colonies developing in plain agar, 1 per cent. acid, at incubator temperature in 48 hours were 50 and 172, respectively. Sample 3 (domestic spring supply), showed no gas production in lactose broth with 1 c.c. and 5 c.c. quantities, and the incubator count was 68 colonies per c.c. I therefore submitted a report stating that the results were inconclusive and advised an epidemiologic investigation of the outbreak and a field study of the water supplies. These were asked for and were made by me during the first 2 days of September with the following results:

On my arrival in McGill I learned from the company's physician that there had been no typhoid fever in the village for more than a year, but that 8 cases had suddenly come to notice between August 9 and 17, a period of 8 days. The company's physician was of the opinion that the milk supply was the source of infection because the dairy was in an insanitary condition and all patients had been using milk from the dairy—the only one in McGill.

Accompanied by the chief surgeon of the Consolidated Copper Company, I visited every patient in order to get all information first hand. The information thus gathered showed that all patients had been getting milk from the only dairy in McGill and all had been supplied with drinking water from the "Domestic Spring" supply. There were no cases on the "Duck Creek" supply, although the families using this water were using the same milk as those on the other water supply. This information, therefore, pointed strongly toward the "Domestic Spring" water as the source of infection because it would be most extraordinary to have typhoid fever developing from the milk supply in those families using water from the "Domestic Spring" supply and not in those using water from the "Duck Creek" supply. This situation was explained to the company's surgeon and their engineer, and a careful inspection of the "Domestic Spring" was recommended. Before making this inspection I was assured by the engineer that the "spring" was tightly enclosed and could not possibly become contaminated.

The inspection, however, revealed a very different state of affairs. The "Domestic Spring" is in reality a shallow well about 20-25 feet in depth and situated in a small gully in the north end of the village. All surface drainage from a considerable area is into the gully and toward the well. In fact, the well is situated in a large natural ditch, as shown in the photograph. The well was lined with planks but these were in a dilapidated condition and formed no barrier against the introduction of surface filth, and rain or snow water. Moreover, a careful inspection of the premises showed that these were used rather freely by tramps in place of a privy. In fact, the heavy timber projecting from the side of the enclosure over the well (see illustration) made this a convenient place for that purpose. Our inspection revealed perfectly fresh deposits of human excrement right at the edge of the well. The filth deposited here could not escape being washed into the well whenever there was a heavy rain.

Summer rains are not of frequent occurrence in this section of the country, but it was learned that there had been a heavy rain about July 31 or August 1.

The cases of typhoid fever began to appear on August 9 and the outbreak was apparently at an end by the 17th. Evidently the immediate surroundings of the well had been polluted by a typhoid carrier shortly before this rain.

A total of eight cases were recognized during the outbreak. These ranged in age from 6-56 years. Four were between the ages of 20-30 years and three were over 30. Six were males and two were females.



Photograph of well responsible for typhoid outbreak at McGill, Nevada.

A second outbreak of more than ordinary interest and which has been definitely traced to its source, occurred in Ely, Nevada, during January, February and March, 1918.

In response to a telegram I went to Ely on March 26, 1918, and in an interview with Dr. W. S. Holmquist, health officer of White Pine County, learned that there had been reported eleven cases of typhoid fever between January 25 and March 26. It was also learned that Dr. Holmquist had made

a careful inquiry into the milk supply of every patient and had found that all had been using milk from a certain dairy "M." The doctor had then visited this dairy and was informed that there had been no sickness, but that they had engaged a new helper in the dairy about January 10. On questioning this individual, it was learned that he had suffered from a severe and protracted attack of typhoid fever during October and November, 1917, and that this was in fact the first employment that he had engaged in since his recovery. On the finding of these facts, Dr. Holmquist ordered the man to leave the employ of the dairy and be confined in the county hospital, awaiting the arrival of the state bacteriologist.

After having been informed of these facts, I visited all patients who had been reported as typhoid cases, in order to gather first hand all information that might throw light on the source of infection.

The data gathered in this investigation showed that all patients had been using city water, and that all were heavy milk drinkers and had been getting milk from dairy "M." The city water was considered safe and this was verified by a bacteriologic analysis of a sample collected on March 27. Practically all of the patients had been eating at their homes and all denied having been away from Ely for over a month prior to the onset of their illness. Groceries, butter, and other supplies for table use were bought at various stores, and it did not appear that they were getting anything in common except the drinking water and the milk. All data gathered, therefore, pointed very strongly to the milk from dairy "M" as being the vehicle of infection. This conclusion was greatly strengthened by the fact that, as stated, one of the helpers who had recently come into the employ of this dairy was convalescent from typhoid fever only about six weeks before entering the employ of the dairy. This man was therefore given a brisk cathartic (magnesium sulphate) to get several specimens of stool for bacteriologic examination. Two specimens of stool and two samples of urine were obtained, and litmus lactose agar plates made from each within an hour after procuring the sample; a portable bacteriologic outfit having been taken along from the laboratory for this purpose. The plates were carried home to the laboratory and incubated for 24 hours. None of the plates from the two samples of urine, nor those from the first stool, which was fairly solid, contained any typhoid colonies. The plates from the second sample of stool, however, which was semiliquid, contained more typhoid colonies than colonies of *B. coli*. These organisms were then positively identified as *B. typhosus* by both cultural tests and agglutination tests with typhoid immune serum.

It was proven, therefore, that the outbreak of typhoid fever occurring in Ely between January 25 and March 26 was caused by the infection of a milk supply by a dairy helper who was a typhoid bacillus carrier.

One lesson that should be learned from the results of this investigation is to the effect that no person should be allowed to enter into the employ of a dairy until his past history relative to typhoid fever has been ascertained by the local health officer. If a history of typhoid

fever is obtained, there should be made a careful bacteriologic examination of the urine and several specimens of stool to determine the presence or absence of typhoid bacilli. The stools must be obtained with the aid of a cathartic or the findings are more likely to be negative, even though the person is a chronic carrier. More trustworthy results could, no doubt, be obtained by making a bacteriologic examination of the duodenal contents, but the facilities for procuring the specimen are generally not at hand in the average country village and small city.

THE EFFECT OF FEEDING YEAST ON ANTIBODY PRODUCTION

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This work has been undertaken in the attempt to discover the effect of feeding yeast on antibody formation in the rabbit, with the view to a possible explanation of the therapeutic value claimed for yeast in infectious diseases.

From the time of Hippocrates until the present day, yeast has been used as a therapeutic agent in diseases, particularly in those of an infectious nature. Hippocrates used it in the local treatment of leukorrhea, and later it was used by the uncivilized tribes of France and Germany in the same way. Clinicians in the second half of the last century were interested in both its use and its mode of action, and we find articles in regard to yeast, its dosage and its action in various diseases, appearing intermittently from the time of Mosse¹ in 1852, to the present work of Hawke² and his associates. The French clinicians in the last years of the last century and the early years of the present, were particularly interested in its mode of action, and such men as Landau, Beylot, Petit, Nobécourt, and others worked on the problem, but obtained no positive results. Hypotheses in abundance were forthcoming, but the true mode of action was unknown. Debousy³ postulated a "vaccine" secreted against the microbe of furunculosis. Landau⁴ thought there might be a threefold action: a direct cellular antagonism, a mechanical expelling of the organisms causing the disease by the overgrowth of the yeast, and finally the use of substratum for its own benefit to the detriment of the other organisms. Beylot⁵ attributed an antiglycemic rôle to yeast. De Backer and Jacquin gave it a phagocytic activity, while Calmette proved that it had no such powers. Petit held that yeast could have no antiseptic power, for all those substances with this power suppress the fermentation of yeast; so that he thought that the most important factor was the vegetable nature of yeast, the fact that it takes up enormous quantities of carbon dioxid and renders the atmosphere toxic for the aerobic bacteria. Vigier⁶ considered ferments, vegetable nature, growth, and all other suggestions unimportant, and he believed that some other substance, an alkaloid for instance, was the substance to which the action of yeast in disease was to be attributed. On the other hand, Coirre⁷ said that the therapeutic activity was always proportional to the

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¹ Lancet, 1852, 2, p. 113.

² Jour. Am. Med. Assn., 1917, 69, p. 1243.

³ Jour. de méd. et de chir. prat., 1894, 65, p. 476.

⁴ Jour. de méd. et de chir. prat., 1896, 67, p. 274.

⁵ Thèse de Bordeaux, 1896.

⁶ Revue de thérap. méd.-chir., 1900, 67, p. 152.

⁷ Revue de thérap. méd.-chir., 1900, 67, p. 82.

energy of the ferment, which remained the sole criterium to serve as a guide, since it is unknown whether the therapeutic value resides in the act of fermentation, or whether the presence of the ferment per se, fighting the germs that it meets, has the desired effect. Nobécourt⁸ suggested the possibility that other as yet unknown substances might be the true active principles of yeast, but concluded that the vital action of the vegetable was the really important action. Bolognesi⁹ did not believe that the zymases themselves were important, but that there was some soluble ferment not precipitated by the alcohol which was the true active portion. Brocq¹⁰ finally concludes that yeast acts as a "modifier of the general state, active enough to render the ground unfavorable for the development of the staphylococcus, but not a specific for it." Lardier¹¹ has shown that as yet no conclusion has been reached by the French school. Hawke and his co-workers have emphasized the efficacy of yeast in the treatment of furunculosis and other staphylococcus infections. They have given no explanation for its action, but suggest that the laxative value combined with some fixed effect on the intestinal tract may be the cause of improvement that follows its use.

Since all the diseases for which yeast is said to be a specific are induced by staphylococcus, streptococcus, and other pyogenic organisms, and since the recovery from these diseases is due to an increased antibody production, it is logical to suspect that yeast, if it has any beneficial action, has an influence on the yield in antibodies. In order to find if this is so, we studied the effect of the feeding of yeast on the production of antibodies to sheep blood cells in rabbits. We had in mind, also, the possibility of discovery and isolation of a substance which specifically increases the production of antibodies, an accomplishment not yet attained, but the importance of which has been realized in clinical medicine. The experiment has been made three times with a series of new animals each time.

Six full-grown rabbits were used each time, two as controls; four were fed with yeast. The controls were fed as much carrots and oats as they desired. The remaining rabbits were placed in individual cages, and each of them fed daily half a cake of Fleischmann's compressed yeast mixed with ground carrots, and after this had been eaten, as much fresh carrots and oats as the animals would eat. After the first time, this was continued for a period of approximately one month.

In the first series, each animal was injected with 30 cc of fresh whole sheep blood intraperitoneally. This was found to be toxic, so the remaining work was done with washed sheep corpuscles. In the first series, 3 to 4 cc of blood was drawn from an ear vein from each animal every 4 days until the sixteenth day, and the antibodies were titrated after each bleeding. In the table, the figures referring to "Lysin" represent the highest dilution of rabbit serum in question in which complete lysis of sheep corpuscles was produced. The tubes were incubated for 2 hours and then placed in the icebox

⁸ Jour. de méd. de Paris, 1900, 12, p. 266.

⁹ Revue de thérap. méd.-chir., 1899, 66, p. 695.

¹⁰ Presse méd. 1899, 1, p. 45; Jour. de méd. et de chir. prat., 1900, 71, 896.

¹¹ Thèse de Paris, 1901-1902.

until morning. In each test the total quantity of the mixtures was 0.6 c.c., of which 0.2 c.c. was a 5% suspension of sheep corpuscles and 0.012 c.c. guinea-pig serum as complement; and the remainder rabbit serum and salt solution. Since the estimation of the lysin was made at various times, and not all at once, the figures must be regarded as only approximately correct with regard to the lysin in the rabbit serum at different bleedings because of the undoubted variations in the activity of the guinea-pig serum used as complement, and in the resistance of the sheep corpuscles employed in the different sets of tests. The results, however, are believed to be accurate enough for the requirements in this case. The figures and curve referring to "Agglutinin" give the highest dilution of rabbit serum in which there was a trace of agglutination, namely a halo around the clumped corpuscles. In the case of "Precipitin" the figure and curve refer to the highest dilution of water laked sheep blood with which the serum of the rabbits formed a definite precipitate after 1 hour at room temperature.

In the remaining experiments, the estimation of lysin and agglutinin was made at the same time so that the concentration of the complement and sheep corpuscles remained the same throughout. The precipitin tests were not made. In the second series of experiments, the animals were bled every 3 days, while in the third series, they were bled every 4 days. The same concentrations were used as in the case of the first group.

From the curves in each case it is seen that in no case is there a higher concentration of hemolysins in the yeast-fed animals than in the controls.

In Table 1 are given the results obtained with the third group of animals which fairly well represent the results obtained with the other groups.

TABLE 1

THE EFFECT OF FEEDING YEAST AFTER THE INJECTION OF SHEEP BLOOD ON THE PRODUCTION OF LYSIN AND AGGLUTININ IN RABBITS

Days after Injection of Antigen	Rabbit 1		Rabbit 4		Rabbit 5—Control		Rabbit 6—Control	
	Lysin	Agglutinin	Lysin	Agglutinin	Lysin	Agglutinin	Lysin	Agglutinin
4	0	192	12	768	6	192	6	192
8	6	1,536	12	1,536	12	3,072	12	1,536
12	12	3,072	12	3,072	24	3,072	24	12,288
16	12	192	96	6,144	96	6,144	96	12,288
20	12	384	96	6,144	96	6,144	96	12,288
24	24	768	48	1,536	96	1,536	96	3,072
28	48	384	48	1,536	48	1,536	48	192
32	0	96	12	192	12	1,537	12	192

The figures under "Lysin" give the highest dilution in which complete lysis of sheep blood was produced. The figures under "Agglutinin" give the highest dilution in which distinct agglutination was produced.

The first series shows that lysin, agglutinin and precipitin formation is greater in control animals than in yeast fed ones over a period of sixteen days. In series 2, lysin formation is considerably greater in the control than in the yeast-fed animals, but the agglutinin formation

is slightly higher in the yeast-fed animals than in the controls. In series 3, the agglutinin formation in both control and yeast-fed rabbits is the same, but in the controls the agglutinin remains in high concentration a longer time in the circulating blood of the control than in the yeast-fed animals. The lysin production is much higher in the control than in the yeast-fed animals.

From our work on the antibodies formed to sheep corpuscles we have been forced to conclude that yeast does not act by increasing antibody formation in the rabbit; in fact, it may even cause a reduction in the amount of antibodies formed as compared with those of the control animals.

Opsonins, the most active antibody infections, were not estimated, but the opsonin curve usually parallels the other antibody curves.

The laxative value of yeast was also found to be lacking, for there was no evidence of it in any of the yeast-fed rabbits of these three series. Their diet remained the same as that of the control animals in every respect save the one additional factor of yeast. We are unable for this reason to agree with Hawke's conclusions in regard to the rôle played by yeast in skin and other diseases; namely, a laxative value combined with a probable fixed effect on the intestinal tract.

The importance of any unknown substances we are not able to discuss. It may be that they are of great importance and play the determining rôle in the favorable effects obtained by yeast. As to alkaloids or other chemical substances, we have felt there is no need or evidence for their consideration.

CONCLUSIONS

The feeding of yeast has no stimulating effect on the production of antibodies to sheep blood in rabbits. The production of antibodies was even less than in controls.

There is no evidence of any effect of yeast on the gastro-intestinal tract of rabbits.

A BIOLOGIC CLASSIFICATION OF HEMOLYTIC STREPTOCOCCI

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INTRODUCTION — GENERAL CONSIDERATIONS

Streptococci as a group have long occupied a position of importance among pathogenic micro-organisms, and their importance has been enhanced in recent years. Their ubiquity and their pathogenicity place them in the front rank in the study of pathogenic bacteria. Yet surprisingly little is known of them. It is known that one large group of streptococci has the ability of hemolyzing blood, and that another large group has not this activity. Furthermore, it is known that their sugar-splitting activities are variable and that, by this criterion and by certain other cultural characteristics, subgroups exist. Aside from these facts very little is known.

A further interest in the streptococcus group, especially in the hemolytic members, was created by the high incidence of infection and healthy carriage of this organism at mobilization camps during the past two years. At some of the army camps and cantonments as high as 20-25% of healthy carriers of hemolytic streptococci were found.¹ These figures are much higher than those formerly obtained for civilian populations. Thus Smillie² found only a small percentage of carriers in a boys' school. A high carrier incidence has, however, been reported from many camps. Cumming, Spruit and Lynch³ found 6% of healthy carriers at Fort Sam Houston; Fox and Hamburger⁴ and Levy and Alexander⁵ found 15% at Camp Zachary Taylor, and as high as 89% among regiments where bronchopneumonia was prevalent. Irons and Marine⁶ report 70% of healthy carriers at Camp Custer.

That recently numerous individuals in civilian groups have harbored the hemolytic streptococcus is indicated by an examination of 76

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¹ Hamilton, C. D., and Havens, L. C.: *Jour. Am. Med. Assn.*, 1919, 72, p. 272.

² *Jour. Infect. Dis.*, 1917, 20, p. 45.

³ *Jour. Am. Med. Assn.*, 1918, 70, p. 1066.

⁴ *Jour. Am. Med. Assn.*, 1918, 70, p. 1827.

⁵ *Jour. Am. Med. Assn.*, 1918, 70, p. 1758.

⁶ *Jour. Am. Med. Assn.*, 70, p. 687.

students at a school in South Carolina whose throat cultures showed 22 to be carriers, or 29%.

This in turn raised a problem of epidemiologic importance. Were all these carriers to be considered a menace or were the majority harboring a relatively avirulent strain? A corollary of this was the determination of the biologic unity or multiplicity of hemolytic streptococci with a view to therapeutic and preventive measures.

Various attempts have been made in the past to differentiate the hemolytic streptococci. One of the best of these has been based on their carbohydrate reactions. Holman's⁷ classification of 6 varieties according to their action on mannite and salicin has been widely followed, but, as will be shown later, only one or two of Holman's strains are commonly encountered.

Another basis of differentiation has been their hemolytic activity. Gay⁸ associates increased virulence with increased hemolytic activity and suggests hemolytic tests, using standard amounts of red cell suspensions and determining the amount of a given culture which produces complete hemolysis. The hemolytic activity of streptococci will be discussed later in this report. At this point it can be said that it is a recognized fact that streptococci vary greatly in virulence, not only among different strains, but also with the same strain, depending on the environment.

A third method of classification, and one which has given rise to the most progress in the study of other micro-organisms, is based on biologic reactions. Kinsella and Swift,⁹ from a study of a limited number of strains, by means of the complement fixation test, came to the conclusion that they were identical. Similarly, Howell¹⁰ finds a low specificity by complement fixation for this group. Davis,¹¹ studying the anaphylactic reactions of hemolytic streptococci, found that a number of different strains interacted. It should be pointed out, however, that in these experiments only a limited number of different cultures were used. Furthermore, the delicacy of the complement fixation reaction and of anaphylactic tests is admittedly not as great nor their specificity as reliable as in agglutination tests, for example, where high dilutions of serum can readily be used.

⁷ Jour. Med. Research, 1916, 34, p. 377.

⁸ Jour. Lab. and Clin. Med., 1918, 3.

⁹ Jour. Exper. Med., 1918, 28, p. 169.

¹⁰ Jour. Infect. Dis., 1918, 22, p. 230.

¹¹ Jour. Infect. Dis., 1917, 21, p. 308.

A further step in the differentiation of hemolytic streptococci has been made by Avery and Cullen.¹² They have studied the final H-ion concentration of cultures of strains of bovine and human origin, and have found a constant and remarkable difference between strains from these two sources. Human strains produced constantly less acid than the bovine strains, i. e., the final H-ion concentration was consistently higher with the latter than with the former group.

Recently it has been shown in a preliminary report that hemolytic streptococci apparently differ among themselves in respect to their serologic characteristics as judged by agglutination tests, bactericidal experiments and protective properties for animals, especially mice. It was shown that a serum high in agglutinins could be produced in rabbits and that about 55% of a series of 110 strains fell in one group whereas the rest showed serologic differences. The purpose of this paper is to test the work presented in the preliminary report, and to describe further experiments in connection with the serologic grouping of the hemolytic streptococci.

Source of Strains.—The basis of these experiments is furnished by a series of strains from different sources which include throat cultures from healthy carriers, from cases of acute bronchitis, from measles patients, and from cases of tonsillitis and sore throat; sputum from pneumonia and lung cultures at autopsy; empyema; gunshot wounds, and one case of renal infection. The number of cultures from each source is presented in the following table:

TABLE 1
SHOWING NUMBER OF CULTURES FROM EACH SOURCE

Throat cultures—all sources.....	179
Healthy carriers	80
Acute bronchitis	60
Measles	9
Tonsillitis and sore throat.....	30
Pneumonia—sputum	20
necropsy	21
Empyema	67
Gunshot wounds	4
Renal infection	1
Total.....	292

In addition, thanks are due to Dr. O. T. Avery of the Rockefeller Institute, who kindly furnished a few representative strains.

Cultures were in all cases first streaked on a blood-agar plate and characteristic colonies showing a definite, clear hemolytic zone at the end of 18-24 hours' incubation were then fished and grown on blood-agar slants. A smear was examined microscopically for morphology, and the slant was kept in the icebox for a stock culture.

¹² Jour. Exper. Med., 1919, 29, p. 215.

These strains may be considered quite representative, since they were obtained not from the population of a single community, but from arrivals in the camp (Wadsworth) from widely scattered parts of the country, and from patients from many camps. Furthermore, the pathologic conditions represented by the series is quite inclusive.

Cultural Characteristics.—The cultural characteristics of hemolytic streptococci have previously been described in detail, and it is necessary only to mention certain of their growth activities which bear on their differentiation. The criterion of any strain of hemolytic streptococcus is, of course, its ability to hemolyze red blood corpuscles. Furthermore, the blood is not only laked, but is destroyed still further. The hemoglobin is broken down so that on a blood-agar plate there is a clear zone containing no pigment. Another point in the differentiation of these organisms by means of their hemolytic action is that the zone is clear-cut—the hemolysin acts completely, if it acts at all.

Among different strains, the hemolytic action varies markedly. In the first place, some colonies produce a wide zone, others a narrow zone, merely a rim around the colony. Again the amount of a 24-hour broth culture which is required to completely hemolyze a given amount of blood (0.5 cc of a 5% suspension of human cells) varies considerably, not only between different strains, but also from time to time in the same strain. Some strains completely hemolyze this amount of blood in 0.05 cc amounts, others do not produce complete hemolysis in 0.5 cc amounts.

As remarked, the hemolytic action of any particular strain seems to vary with its virulence. Streptococci rapidly decrease in virulence with growth on artificial mediums. At the same time its hemolytic action diminishes. A strain of hemolytic streptococcus grown for 2-3 weeks on blood agar possesses only feeble hemolytic power and such a culture, injected into mice, may fail to kill in 2 cc amounts. Its virulence, however, can be quickly raised so that after 6 passages, in the case of 3 strains studied, 0.1 cc of a 24-hour culture killed invariably in less than 24 hours. Along with increased virulence its hemolytic power rises. Its virulence is lost with equal rapidity, cultivation for from 7-10 days on artificial mediums again reducing it to its former avirulence and its feeble hemolytic activity.

Carbohydrate tests with different strains of hemolytic streptococci, as mentioned, have been used for their differentiation. However, of the 6 groups which have been determined by this method only two are commonly met with. These are *S. anginosus* and *S. pyogenes*, the former splitting only lactose, the latter acting on salicin as well, neither affecting mannite. Of 120 strains of this series studied in this way, 80% split only lactose, the other 20% acted on both lactose and salicin. In this series, no evidence of any relation of the action on sugars to the hemolytic action, to the serum reactions, or to the virulence of the organisms could be found.

It can be seen from the preceding observations that no method was at hand for satisfactorily differentiating hemolytic streptococci. Its lack has been felt. It is known that animals can be immunized against individual strains of streptococci, but this has acquired no practical importance in spite of repeated attempts. No adequate basis for determining different strains has been available. The method in practice for producing antistreptococcus serum has been to collect as large a number of different cultures as possible and to trust that they con-

stituted a fairly complete representation of the streptococci. Consequently, the serum was either of low titer due to its great polyvalency or was useless because it did not contain antibodies against the particular strain causing the infection. The same criticism applies equally to the vaccines made in this way. Hence, a method for "typing" hemolytic streptococci is for this reason alone of practical importance.

Serologic Experiments.—Three cultures were chosen at random from the series and were used for immunizing rabbits. The cultures were grown for from 3-4 weeks on blood agar and were avirulent for rabbits. The injections were made with the unheated centrifuged sediment of serum broth cultures resuspended in normal salt solution. The avirulent living organisms were injected intravenously in increasing amounts, on 3 successive days, followed by a 3-day interval. Nine such injections were, in most cases, sufficient to obtain an agglutinin titer of 5,000-10,000.

A plain or glucose broth culture of hemolytic streptococcus has a tendency to clump spontaneously. It was found, however, that plain broth to which 10% defibrinated blood has been added gives a medium in which the organisms grow rapidly and which in 12-16 hours shows a uniform, even turbidity. The blood is only very slightly hemolyzed by old stock cultures in this length of time, the hemolysis, such as there is, being confined to the butt of the tube, leaving the upper portion free from hemoglobin. Tubes were set up containing dilutions of serum from 1:10-1:4,860 in 0.5 cc amounts and 0.5 cc of culture making 1 cc, the total amount. A control tube was used with each culture containing 0.5 cc salt solution and 0.5 cc culture. The tubes were incubated at 37 C. for 4 hours and then placed in the icebox over night and the final reading made the next morning, the titer given in each case being the highest dilution of serum in which complete agglutination took place.

The serum obtained from rabbits injected, as outlined, with these 3 strains was used in agglutination tests with all the cultures of the series. It was soon found that these 3 serums were identical, that is, strains which agglutinated with one, agglutinated with the other two, and strains which failed to agglutinate with one were not agglutinated by the others. Consequently the serums were pooled. With this serum 139 strains, or 47%, of the 292 members of the series were agglutinated in dilutions of 1:1,000 or higher. The other 153 strains failed to agglutinate, or did so only in low dilution—never higher than 1:100, with the exception of a small series which will be discussed in detail later.

One of these strains which failed to be agglutinated was then used for the production of an immune rabbit serum, using the technic employed for the first serum. With this serum 54, or 19%, were found to agglutinate. With the exception of the series mentioned above, no instances of cross-agglutination were found. Thus, according to their agglutination reactions, we now have two groups which are distinct and well defined. They include together 191, or 66%, of the 292 members of the series.

A third group comprising 79 members of the series, or 27%, was obtained by similar methods. This group, like the others, contains a few strains which show evidence of a certain interrelation as judged by cross-agglutination, but the majority are distinct.

It appears, then, that by means of agglutination tests three distinct groups of the hemolytic streptococci are established, constituting 93%

of this series of 292 strains. The first group is much the largest (47%), the second group contains 19%, and the third, 27%. The remaining 22 strains, or 7% of the series, evidently is a heterogeneous group, possessing different biologic characteristics. At this time no evidence can be presented to show that this group can be satisfactorily subdivided.

In order to determine whether other immune bodies, aside from agglutinins, corroborated this grouping, bactericidal tests were made on a considerable number of strains. Each test was made with 4 tubes: To each tube was added 0.5 cc of a blood broth culture of the particular strain to be tested and 0.1 cc of fresh guinea-pig serum as complement. To the first tube was added 0.5 cc group 1 serum, to the second tube 0.5 cc group 2 serum and to the third tube 0.5 cc serum 3. The fourth tube received 0.5 cc salt solution as a control. The tubes were then incubated for 4 hours at 37 C. and 0.1 cc of the mixture in each tube was plated in blood agar. After 24 hours' incubation, the number of colonies were counted. The following table shows a comparison of bactericidal tests with agglutinations.

TABLE 2
COMPARISON OF AGGLUTINATION TESTS AND BACTERICIDAL EXPERIMENTS

Number of Culture	Number of Colonies*				Agglutination Tests		
	Serum 1	Serum 2	Serum 3	Salt Solution	Serum 1	Serum 2	Serum 3
139	0	1,000	1,000	1,000	4,860	180	10
161	0	200	100	200	4,860	20	40
170	0	100	100	200	4,860	180	60
164	0	200	200	200	4,860	20	10
163	50	1,000	1,000	1,000	4,860	20	60
169	25	1,500	2,000	2,000	4,860	180	60
172	100	1,000	1,000	2,000	4,860	10	60
186	0	100	200	200	4,860	60	20
79	10	200	400	500	4,860	180	10
3	0	500	500	500	4,860	10	10
33	10	500	1,000	1,000	10,000	180	10
50	100	2,000	2,000	2,000	4,860	10	10
124	0	500	400	400	5,000	60	180
117	500	10	500	500	10	4,860	20
206	200	0	200	200	60	4,860	60
51	1,000	10	1,000	1,000	10	4,860	10
67	200	20	300	500	180	4,860	10
129	1,000	100	2,000	2,000	1,620	4,860	10
152	200	0	200	200	60	4,860	20
180	200	25	200	250	180	4,860	10
245	100	0	100	200	10	4,860	20
121	100	20	200	200	180	1,620	540
118	200	0	500	500	10	4,860	60
196	1,000	0	500	1,000	10	4,860	10
135	1,000	1,000	10	1,000	10	10	4,860
183	1,000	2,000	50	2,000	540	60	4,860
178	2,000	2,000	100	2,000	10	60	4,860
151	1,000	500	10	2,000	180	10	4,860
211	200	200	0	200	20	10	4,860
209	100	100	10	200	10	10	4,860
218	200	100	0	200	60	180	4,860
248	1,000	2,000	100	2,000	10	10	4,860
34	1,000	1,000	10	2,000	20	60	4,860
16	500	500	50	500	50	10	4,860
199	200	200	0	500	1,620	1,620	4,860

* After 24 hours incubation on a blood-agar plate inoculated with 0.1 cc of serum culture incubated for 4 hours.

It can be seen from the table that the bactericidal experiments agree with the agglutination tests; that is, strains were killed by the same serum with which they agglutinated, but not by serum of another group.

It was mentioned that a number of strains seemed to be interrelated in that, while with one serum they agglutinated in dilutions of 1:5,000, they also agglutinated with serum of another group in quite high dilution, as high as 1:1,500 in a few instances. These strains are interesting in that they furnish evidence of a lack of complete and clear cut differentiation between the groups, such as exists between the pneumococcus groups. One group appears to shade into another somewhat as the different strains of meningococcus are interrelated. The strains which agglutinated higher than 1:100 with more than one serum are summarized in table 3, together with their agglutination titer with each serum. With serum of the titer used in these tests (1:5,000 or 10,000) a positive agglutination in dilutions of less than 1:100 was regarded as nonspecific.

TABLE 3
INTERRELATED STRAINS SHOWING AGGLUTINATION TITER WITH EACH GROUP SERUM

Number of Culture	Serum 1	Serum 2	Serum 3
3	10,000	10	180
10	4,860	10	180
33	10,000	180	10
35	4,860	10	180
60	3,240	540	180
79	4,860	180	10
107	3,240	10	180
124	5,000	60	180
146	4,860	180	180
159	4,860	180	10
154	3,240	180	60
162	4,860	10	540
179	4,860	60	180
191	4,860	1,620	10
170	4,860	180	60
209	4,860	540	10
58	180	4,860	60
129	1,620	4,860	10
121	180	1,620	540
170	1,620	4,860	20
180	180	4,860	10
235	540	4,860	10
96	180	4,860	10
207	20	4,860	180
199	1,620	1,620	4,860
95	540	540	4,860
125	180	180	4,860
151	180	10	4,860
183	540	60	4,860

An analysis of table 3 shows that 29 strains indicate by their agglutination an interrelation between the groups. Sixteen members of group 1 which are agglutinated in high dilution by their homologous

serum were agglutinated in dilutions of 1:100 or higher by one or both of the other two serums. Seven were agglutinated only by serum 2 in addition to the serum of their own group in dilutions varying from 180-1,620 and 7 only by serum 3 in dilutions of 180-540. Two strains, 60 and 146, were agglutinated by both serums 2 and 3 in dilutions above 1:100. Eight strains of group 2 showed some relation to the other 2 groups; 6 only to group 1, and 1 only to group 3. Strain 121, in addition to agglutinating in its homologous serum up to 1:1,620, agglutinated in serum 1, 1:180, and in serum 3, 1:540. Five members of group 3 were agglutinated by serums 1 and 2 in dilutions above 100. Two were agglutinated by serum 1, 1:180 and 1:540, respectively, and 3 equally by serums 1 and 2, 1:1,620, 1:540 and 1:180.

Thus 29, or about 10%, of the 292 strains gave evidence from agglutination tests of some relation to other groups besides the one with the serum of which they were agglutinated in highest dilution. This relationship is borne out to some extent by bactericidal experiments; as, for example, in table 2, culture 79, which is agglutinated by serum 1, 1:4,860, by serum 2, 1:180 and serum 2, 1:10, shows by bactericidal tests, 10 colonies with serum 1, 200 with serum 2, while serum 3 showed scarcely more effect than normal salt solution, namely, 400 colonies. Cultures 33, 124, 129, 183 and 199 are further examples. This relationship is not, however, borne out in all cases by bactericidal tests, as for example, strain 121, which was agglutinated by serum 3, 1:540, gave as many colonies in the bactericidal test with this serum as the control. Cultures 159, 170, 180, and 151 are further examples.

While agglutination tests and bactericidal experiments give this evidence of interrelationship among groups, protective experiments with mice fail to show it. Only the serum which possesses a titer above 1,000 for the strain used for injection seems to possess sufficient protective properties to prevent death with a lethal dose. These experiments will be described in detail.

From several points of view a study of this series of hemolytic streptococci grouped according to the character of the infection or the pathologic condition from which the strains were isolated is interesting and instructive. The following tables show the groupings of these strains according to the character of the infection. Table 4 shows the groupings of the whole series for comparison with the following tables.

TABLE 4
SOURCES

Total Strains from all Sources:			5. Pneumonia—Sputum:		
Group 1.....	137	47%	Group 1.....	8	40%
Group 2.....	54	19%	Group 2.....	4	20%
Group 3.....	79	27%	Group 3.....	6	30%
Group 4.....	22	7%	Group 4.....	2	10%
Total.....	—292		Total.....	—20	
1. Throat Cultures—Healthy Carriers:			6. Pneumonia—Necropsy:		
Group 1.....	37	46%	Group 1.....	20	95%
Group 2.....	16	20%	Group 2.....	0	0%
Group 3.....	18	22%	Group 3.....	1	5%
Group 4.....	9	12%	Group 4.....	0	0%
Total.....	—80		Total.....	—21	
2. Throat Cultures—Acute Bronchitis:			7. Empyema:		
Group 1.....	29	48%	Group 1.....	28	42%
Group 2.....	12	20%	Group 2.....	15	22%
Group 3.....	14	23%	Group 3.....	20	30%
Group 4.....	5	9%	Group 4.....	4	6%
Total.....	—60		Total.....	—67	
3. Throat Cultures—Measles:			8. Gunshot Wounds:		
Group 1.....	5	55%	Group 1.....	0	0%
Group 2.....	4	45%	Group 2.....	0	0%
Group 3.....	0		Group 3.....	3	75%
Group 4.....	0		Group 4.....	1	25%
Total.....	—9		Total.....	—4	
4. Throat Cultures—Tonsillitis and Sore Throat:			9. Renal Infection:		
Group 1.....	9	30%	Group 1.....	1	
Group 2.....	3	10%			
Group 3.....	17	57%			
Group 4.....	1	3%			
Total.....	—30				

It will be seen, in the first place, that the group of 80 strains from carriers compares very closely with the percentages of the entire series. This would appear to be of epidemiologic importance in that healthy carriers are a source for any and all of the pathologic conditions that are caused by hemolytic streptococci. From the small evidence at hand the impression has been gained that carriers of these organisms rarely develop an infection, at least with the particular strain which they harbor.

The strains obtained from cases of bronchopneumonia, while small, are exceptionally interesting. From the table it is evident that the percentages of each group follow approximately the percentages of the whole series. No one group seems to be associated predominantly with this condition. This is the case with antemortem specimens. Postmortem cultures, however, show a great contrast to the cultures from sputum. Of 21 strains obtained from necropsies, all but one were found to belong to the first group, the other one falling in group 3. Although it is hazardous to draw conclusions from a small series such as this, yet the contrast between the antemortem and the postmortem

cultures is so marked that it would appear to indicate a special virulence for group 1 organisms. While all the groups may cause pneumonia in a proportion relative to their frequency, it would seem that the prognosis is much more favorable in group 2 and 3 infections than in the case of group 1. A further point in connection with streptococcus pneumonia is of interest. It appears that no one group predominates in the causation of empyema. This condition is as likely to develop following infection with one group as with another. The proportions of the groups in the strains obtained from empyema correspond closely with the groups in pneumonic sputum.

The series of cultures from cases of tonsillitis and sore throat is also of interest. It would seem that group 3 is more commonly found in this condition than any other group. But most of these cases occurred in one ward, in fact, all the group 3 cases developed in this one ward, and very probably spread from one source. However this may be, it is interesting from an epidemiologic viewpoint. As a matter of fact, in most epidemics in the past, a common source of the infection has been suspected, but it has not been susceptible of proof. In this small epidemic which occurred in a hospital ward, all but three of the cases were due to a group 3 organism. However, the fact that there were three cases due to other groups—two to group 1 and one to group 4—shows that while most of the cases were due to one source, in all probability not all were developed from the same strain. The mere finding of hemolytic streptococci does not implicate them in the causation of an epidemic, for they may belong to a different group from the one causing the epidemic.

Another point in connection with the epidemiology of hemolytic streptococcus infections is the series of strains isolated from wounds which, while only 4 in number, show only one case which falls into group 4—the heterogeneous group of nonagglutinating strains. A priori, it might seem that most of these strains which infect wounds would fall in a different category from those which are more obviously from a human source, but evidently these organisms, carried into the wound, usually on bits of clothing, belong to the same groups as the strains found in other infections.

A summary of the entire series giving the number of strains according to their source and the number and percentage of each group is collected in table 5.

TABLE 5
SUMMARY OF 292 STRAINS OF HEMOLYTIC STREPTOCOCCI GROUPED BY AGGLUTINATION AND
PATHOLOGIC SOURCE

Pathologic Condition from which Strains Were Isolated	Group 1		Group 2		Group 3		Group 4	
	No. Strains	Per Cent.	No. Strains	Per Cent.	No. Strains	Per Cent.	No. Strains	Per Cent.
Throat Culture:								
Healthy carriers.....	37	46	16	20	18	22	9	12
Acute bronchitis.....	29	48	12	20	14	23	5	9
Measles.....	5	55	4	45	0	0	0	0
Tonsillitis and sore throat.....	9	30	3	10	17	57	1	3
Pneumonia:								
Sputum.....	8	40	4	20	6	30	2	10
Necropsy.....	20	95	0	0	1	5	0	0
Empyema.....	28	42	15	22	20	30	4	6
Gunshot wound.....	0	0	0	0	3	75	1	25
Renal infection.....	1	100	0	0	0	0	0	0
Total.....	137	47	54	19	79	27	22	7

PROTECTIVE EXPERIMENTS

It can be assumed from the experiments previously described that hemolytic streptococci fall into certain quite specific groups and that, in vitro, they are killed by their homologous serum, but not by the serum of other groups. It remains to determine whether they react in the same manner in the animal body and whether increased virulence is accompanied by any change in the serologic reactions of the organism.

In the experiments about to be described the serum used was prepared in the same way as for the agglutination and bactericidal tests, namely, by injecting rabbits with live avirulent organisms, bleeding them when the agglutinin titer for the homologous strain reached 5,000-10,000. Mice were used for the tests and the strains were increased in virulence by passage through mice until 0.1-0.25 cc of a 24-hour broth culture killed in 24 hours. Twelve mice were injected intraperitoneally with the serum prepared in this way, four with the serum of each group, in varying amounts, and at the same time with twice the minimum lethal dose of the corresponding organism. No evidence of any protection by the serum was obtained, whether injected some time before the streptococci or at the same time. Two possibilities were to be considered in consequence. Either the agglutinin titer gave a wrong impression of the protective strength of the serum or the individual strains became altered in their biologic properties as their virulence increased. Both possibilities might be the case. It seemed more probable, however, that the agglutinin content of the serum was not a criterion of the amount of protective action which it possessed. Evidence that this was the case was furnished by the fact that a given strain showed no difference in its agglutinating properties after passage through mice. It gave the same titer with each group serum after several passages through mice as it did when it had been grown for a long period on artificial medium.

The method of producing the serum was consequently modified. Rabbits were given 3 series of injections with avirulent organisms as before, on 3 successive days, with 3-day intervals, making 9 injections in all. Instead of now testing and bleeding as in the case with the serum used for agglutinations, the rabbits received 2 more series of injections—6 on successive days divided by a 3-day interval—of strains which had recently been passed through mice and which were highly virulent for this animal. Using serum prepared in this way the following experiments were carried out:

Mouse 1: (Control.) 0.1 cc 24-hour blood-broth culture 197 (group 1). Dead in 24 hours.

Mouse 2: 0.2 cc 24-hour blood-broth culture of strain 197 + 0.5 cc group 1 serum. Normal 24 hours later.

Mouse 3: 1 cc group 1 serum followed 1 hour later by 0.2 cc 197. Inactive and sick 18 hours later. Peritoneal puncture shows many polymorphonuclear leukocytes loaded with streptococci. Given 0.5 group 1 serum. Normal 24 hours later.

Mouse 4: (Control.) 0.1 cc blood-broth culture of strain 209 (group 1). Dead 18 hours later.

Mouse 5: 0.2 cc strain 209 + 0.5 cc serum 1. Normal 24 hours later.

Mouse 6: (Control.) 0.1 cc 24-hour blood broth culture of strain 170 (group 2). Dead in 24 hours.

Mouse 7: 0.2 cc strain 170 + 0.5 cc serum 2. Normal 24 hours later.

Mouse 8: (Control.) 0.1 cc 24-hour blood-broth culture of strain 135 (group 3). Dead 24 hours later.

Mouse 9: 0.2 cc strain 136 + 0.5 cc serum 3. Normal 24 hours later.

The protocols show that it is possible to raise the protective properties of antistreptococcus serum to a point where it has some value for mice, at least. In the case of mouse 3 the serum apparently had a distinct therapeutic value even after the infection had gained headway and had become systemic.

The following protocols further corroborate the specificity of these 3 groups of hemolytic streptococci. Mice were inoculated intraperitoneally with 2 lethal doses of each strain together with 0.5 cc of each group serum.

Mouse 1: (Control.) 0.1 cc 24-hour blood-broth culture of strain 209 (group 1). Dead in 20 hours.

Mouse 2: 0.2 cc 24-hour blood-broth culture 209 (group 1) + 0.5 cc serum 1. Normal 24 hours later.

Mouse 3: 0.2 cc culture 209 (group 1) + 0.5 cc group 2 serum. Dead in 18 hours.

Mouse 4: 0.2 cc culture 209 (group 1) + 0.5 cc group 3 serum. Dead in 24 hours.

Mouse 5: (Control.) 0.1 cc 24-hour blood-broth culture of strain 170 (group 2). Dead in 18 hours.

Mouse 6: 0.2 cc strain 170 (group 2) + 0.5 cc group 1 serum. Dead in 24 hours.

Mouse 7: 0.2 cc culture 170 + 0.5 cc group 2 serum. Normal 24 hours later.

- Mouse 8: 0.2 cc culture 170 + 0.5 cc group 3 serum. Dead in 18 hours.
Mouse 9: (Control.) 0.1 cc 24-hour blood-broth culture of strain 135 (group 3). Dead in 24 hours.
Mouse 10: 0.2 cc culture 135 (group 3) + 0.5 cc group 1 serum. Dead in 24 hours.
Mouse 11: 0.2 cc culture 135 (group 3) + 0.5 cc group 2 serum. Sick in 24 hours. Dead in 28 hours.
Mouse 12: 0.2 cc culture 135 (group 3) + 0.5 cc group 3 serum. Normal 24 hours later.

In these experiments it is seen that only the homologous serum protected against infection. In each case the mouse injected with the serum corresponding to the group to which the strain inoculated belongs, lived. All the other mice died. However, each mouse received two fatal doses of the culture. In order to determine if the serum of other groups possessed protective properties in any significant amount whatever, the following series of mice were injected with serum and culture mixtures, but the mice receiving heterologous serum were given only one lethal dose instead of two, which the mice injected with homologous serum received.

- Mouse 1: (Control.) 0.1 cc strain 209 (group 1) intraperitoneally. Dead in 24 hours.
Mouse 2: 0.2 cc strain 209 (group 1) + 0.5 cc group 1 serum. Normal 24 hours later.
Mouse 3: 0.1 cc strain 209 (group 1) + 0.5 cc group 2 serum. Dead in 24 hours.
Mouse 4: 0.1 cc strain 209 (group 1) + 0.5 cc group 3 serum. Dead in 20 hours.
Mouse 5: (Control.) 0.1 cc strain 170 intraperitoneally. Dead in 18 hours.
Mouse 6: 0.1 cc strain 170 (group 2) + 0.5 cc group 1 serum. Dead in 18 hours.
Mouse 7: 0.2 cc strain 170 (group 2) + 0.5 cc group 2 serum. Normal 24 hours later.
Mouse 8: 0.1 cc strain 170 (group 2) + 0.5 cc group 3 serum. Dead in 30 hours.
Mouse 9: (Control.) 0.1 cc strain 135 (group 3) intraperitoneally. Dead in 26 hours.
Mouse 10: 0.1 cc strain 135 + 0.5 cc group 1 serum. Dead in 24 hours.
Mouse 11: 0.1 cc strain 135 (group 3) + 0.5 cc group 2 serum. Dead in 24 hours.
Mouse 12: 0.2 cc strain 135 (group 3) + 0.5 cc group 3 serum. Normal 24 hours later.

As in the preceding experiments, the culture injected with heterologous serum killed the mice as rapidly as in the case of the control mice which received no serum. No evidence of any protection by the heterologous serum was obtained. Thus, in view of these experiments, the specificity of the 3 groups of hemolytic streptococci which have

been described is borne out by protection experiments in mice as well as, *in vitro*, by agglutination and bactericidal tests.

DISCUSSION

Many species of bacteria which are identical when studied by cultural and morphologic methods have been found to be composed of a number of groups which are only determined by their biologic differences. When studied by serologic methods these groups are found to be more or less distinct. This comparatively recent method of differentiating bacteria which are otherwise similar has been productive of important advances in the knowledge and in the treatment of infectious diseases. The meningococci and the pneumococci are familiar examples of this fact. Until the various biologic groups of the former were carefully investigated the serum treatment of meningitis due to this organism was much less satisfactory than it has since become. To a lesser extent and in a degree which up to the present has not been productive of such practical results, the typhoid-colon group has been subdivided into a number of serologic groups.

The hemolytic streptococci, then, are not unique among the bacteria. On the other hand, it might be assumed that distinct groups exist in respect to their biologic properties from the fact that various strains present differences in their cultural characteristics and from their parasitic nature. From the present study of a quite representative series of strains of this organism it would seem that this is so.

The value of agglutination reactions in the study of streptococci has been a disputed point. Thus Kligler¹³ has expressed the belief that agglutination tests cannot be depended on in the classification of streptococci. On the other hand, Smith and Brown¹⁴ have used this reaction to demonstrate the identity of different organisms isolated during the course of an epidemic.

However this may be, a practical and simple method of studying the agglutinability of hemolytic streptococci appears now to be available. The results obtained on the basis of agglutination tests have been checked, not only by bactericidal tests, but by protective experiments as well, in the living body. The fact that these last two methods which are less susceptible to erroneous interpretation corroborate the evidence obtained from agglutinations is strong evidence in favor of the reliability of this method in the classification of streptococci.

¹³ Jour. Infect. Dis., 1915, 16, p. 327.

¹⁴ Jour. Med. Research, 1915, 31, p. 455.

The universality of the strains which form the basis of this study supports the assumption that they constitute a representative series. It can at least be assumed that a significant number of these strains fall into definite groups, a number so large that it cannot be disregarded in a consideration of the hemolytic streptococcus. The greatest value of the study of this series lies in a strong argument for the classification in the future of all infections due to this organism.

The finding of evidence that about 10% of the strains of this series show more or less relationship to other groups than the one to which they obviously belong points to the conclusion that the groups of the hemolytic streptococci as herein determined are not so clear-cut as the pneumococcus groups, but yet they appear to be somewhat more definite than the various strains of meningococci which grade into one another by a series of intermediate strains. The great majority of hemolytic streptococci seem to fall into these 3 fairly definite groups, as is clearly evidenced by the protection experiments with mice. Less evidence of an interrelationship is furnished by bactericidal experiments than by agglutination tests and animal inoculations show this to an even less degree.

It has been shown that these 3 groups constitute 93% of this series of 292 strains. The remaining 7% seem to represent strains which possess no biologic properties in common and which consequently cannot be grouped by the methods used in determining the relationship of the other 93%. They seem to be somewhat analogous in this respect to the strains of type IV pneumococci. Whether this is the case or whether they represent inagglutinable strains cannot be stated definitely at present. Judged from the agglutination test, they do not represent an intermediate group or groups, since none of the three group serums affects them in even the lowest titer.

A classification of the strains of this series by their pathologic source brings out several interesting points. The importance of epidemiologic evidence which may be gained from a study of epidemics due to hemolytic streptococci by the methods described is illustrated by the study of a small epidemic of sore throats and tonsillitis which occurred in a hospital ward and, as was shown, the great majority of infections were due to group 3 organisms, suggesting a common source. The comparison of the antemortem pneumonia cultures (sputum) with the series of lung cultures obtained at necropsy suggest a prognostic importance for infections with group 1, as compared with the other

groups, which is rather significant. If the evidence represented by these two small series is borne out by further investigation, the high mortality of streptococcus infections may be explained, since group 1 is much the largest group and consequently is the commonest infecting organism.

The experiments dealing with the protective properties of the serum of each group indicate a further practical reason for the classification of these groups. Each group serum, as was seen in the protocols, protected against infection with its own strains, but afforded no protection whatever against strains of other groups. If specific therapy of hemolytic streptococcus infections is possible, it would seem that it can be successful only on the basis of the consideration of the group to which the infecting organism belongs.

In spite of these considerations, the practical importance of the classification of the hemolytic streptococci must at this time be regarded as problematical. No surmise as to whether these groups will furnish epidemiologic data which will aid in the prevention of infections with this organism or whether therapeutic serums can be produced which will be of value in combating such infections can of course be made at this time. However, a glance at the previous chaotic condition of our knowledge of these comparatively common infections shows how insecure was the basis of any attempt at serum or vaccine therapy in the past. With the foundation which a knowledge of a possible classification of hemolytic streptococci furnishes, future attempts toward the prevention and treatment of these infections can be more intelligent and, in consequence, progress should be more rapid.

SUMMARY

Hemolytic streptococci can be classified by means of practical serologic tests.

From a study of 292 strains from widely varied sources, 3 distinct groups, constituting 93% of the series, have been determined by means of such tests.

Diagnostic serums can be produced for each group, and all infections due to hemolytic streptococci should be classified.

A protective serum for mice against each of these 3 groups has been demonstrated. Each serum is specific for its own group, furnishing no protection against other groups.

AN EPIDEMIC OF WATER-BORNE DYSENTERY

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On Aug. 28, 1918, it was brought to the writer's attention that diarrhea of a very violent type was attacking the inhabitants of Bertrichamp, a town of about 2,000 population, in the department of Meurthe-Moselle, France.

Investigation brought out the following facts: 1. The symptoms of the disease were practically identical in all cases where it was possible to obtain information. Enteric pains were soon followed by diarrhea. Stools were frequent and contained large quantities of blood and mucus which persisted in the feces from two to eight days in the various cases. Passages were accompanied by considerable tenesmus. No vomiting was reported. A rise in temperature to from 99.5 to 100.5 F. was noted in all cases, as well as marked increase in the pulse rates. Several patients seemed apparently on the way to recovery when a sudden collapse, followed by fatal termination of the disease, occurred. 2. Lack of ventilation, and large numbers of flies were noted in all rooms where patients were found. 3. Persons attacked varied in age between 5 and 69 years. 4. No patient had drunk milk or eaten uncooked vegetables for some time prior to the onset of the disease. 5. All patients but two admitted having drunk water from the public supply a short time, usually two or three days, before the first attack. The two who did not admit having drunk water were young children whose evidence was not entirely trustworthy.

The usual precautions were immediately taken to prevent further spread of the disease. Feces were sterilized and buried, flies were driven out, ventilation and cleanliness insisted on. On August 29 the order was given to boil all water used.

Possibly it may not here be out of place to note that a Frenchman will admit drinking water only very reluctantly. For epidemiologic purposes this has a double meaning: Any water history, although more difficult to obtain, will be very accurate; more important, perhaps, any water-borne epidemic will be less widespread in a French town of given population than in an American town having an equal number of inhabitants.

Cases appeared on the following days:

August 18.....	5	
August 19.....	1	
August 20.....	1	
August 21.....	4	
August 24.....	4	
August 25.....	1	
August 27.....	2	
August 28.....	1	
August 29.....	2	
Date undetermined.....	1	
September 4.....	1	
September 8.....	2	
September 9.....	2	Last cases to appear.
Total.....	27	

Among the cases, 7 appeared in families which had a previous case of the disease. Of the 7, five appeared after August 24, so may be regarded as possible contact developments.

Of the cases which developed during September, two had visited friends sick of the disease, and two had ignored the order to boil water before using. The patient who developed diarrhea September 4 had used only boiled water since August 29 and had visited no one.

Cultures from feces of patients showed a nonmotile, gram-negative bacterium having the morphology of *B. dysenteriae*, which gave the growth and reddening characteristic of *B. dysenteriae* and *B. typhosus* on Russell's double sugar medium, and which failed to agglutinate with an antityphoid serum of known potency; because of complicating factors, it was not possible to make direct agglutination tests for *B. dysenteriae*.

Treatment given patients by the civilian doctor in attendance consisted in an almost total restriction of diet, three or four tablespoonfuls of sugar-water being given during the day. In only two cases was antidysentery serum given—both times after the disease had been running several days—and then only in very limited amounts.

Deaths among civilian patients occurred on the following days:

August 25.....	4	
August 26.....	2	
August 27.....	1	
August 29.....	1	
August 30.....	1	Last death.
Total	9	

The death rate, as will be noted, was 33 per cent. The probable reasons for this high mortality lie in the poor physical conditions of

the patients, consequent on four years of poor nutrition and bad living conditions; and in the insufficient treatment given those sick of the disease.

During the period from August 20 to August 30 one French and six American soldiers suffering from diarrhea of the same type were admitted to the military hospitals of this area. Investigation showed that these men had all been quartered in Bertrichamp. Each gave a history of having drunk untreated water from fountains on the main source of supply for the town from two to four days before coming to the hospitals. Cultures from feces were made and *B. dysenteriae* Flexner found in five of the cases.

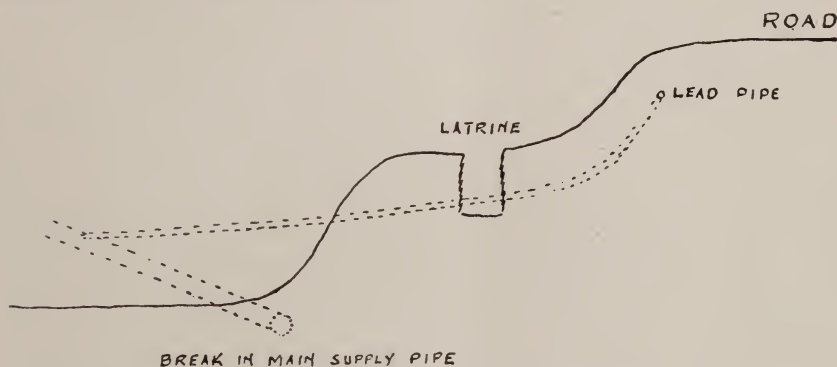


Fig. 1.—Break in main supply pipe from which water flowed saturating soil about latrine and causing contamination of drinking water.

Among the soldiers the disease was not so severe as among the civilians. Recoveries were more rapid and there were no deaths. The better nourishment and treatment is doubtless responsible for this condition.

The water supply of Bertrichamp is of the low-pressure, continuous-flowing-fountain type frequently found in the villages of the Vosges mountains. There are four separate sources from which water is brought into the town by terra-cotta mains. Each of these distribution systems supplies fountains in a different section of the town and is in no way connected with the other sources. The main supply (on which are 10 of the 16 fountains of the town) comes from springs in the forest about two miles back of the town.

A bacteriologic examination of water from each of the fountains of the town was made on August 28. These examinations showed that three of the sources of supply were excellent, but that on the main

source of supply for the town all of the fountains save one gave water which was badly contaminated. This fountain, from which the water was excellent, was the first on the pipe coming from the springs, or the one nearest the source of supply, higher than and perhaps a quarter of a mile from the next fountain on this same pipe line. Samples from the springs showed the water to be uniformly excellent. Samples taken on the two succeeding days confirmed the first analyses. Fountains designated by the patients as those from which they had drunk were all on the supply line from which the water was contaminated.

These analyses indicated a point of contamination between the first and second fountains on the main supply line. Search revealed a break in one joint of the pipe. This joint was directly behind, 15 feet from and 12 feet lower than a latrine dug into the downward sloping bank at the edge of the road. The latrine had been used for three months by passing troops, and had been filled with earth on August 27. A lead pipe which supplied the second fountain on this line was set into the main supply line about 30 feet above the break and ran diagonally up the bank, passing directly behind the latrine and under the road. Steps leading to the latrine exposed this pipe at a point about 6 feet beyond and 2 feet higher than the top of the latrine. Here the lead pipe had been cut and was flowing about 3 gallons per minute, thus causing the earth around the latrine and between it and the main pipe to be thoroughly saturated with water. Fecal matter, practically fresh and undecomposed, was found in veins running about and over the main supply pipe. The main supply pipe was repaired and the condition finally corrected on September 6.

Military reasons made it necessary for the writer to leave this section about a week later. At the time of departure all of the patients had recovered or were doing well. Reports from the secretary of the town showed that no new cases developed later in September or during October, nor were there any further deaths.

CONCLUSIONS

The causative factor of the epidemic reported was *B. dysenteriae* of the Flexner type.

The principal carrier of infection in the above epidemic was water from the main source of supply for the town of Bertrichamp.

ON THE PROTECTION AGAINST THE ACTION OF ULTRAVIOLET LIGHT AFFORDED TO ALEXIN AND SENSITIZER BY CERTAIN SUBSTANCES *

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That ultraviolet light possesses a highly destructive action on various immune bodies — alexin, lysins, agglutinins, toxins and antitoxins, as well as on protoplasm in general, when these substances are exposed either alone or together with certain fluorescent substances¹ such as eosin and fluorescein, has been known for some time. The investigations of Huber² and Busck,³ and particularly those by Baroni and Jonesco-Mihaiesti⁴ and by Abelin and Stiner⁵ with immune substances bear out this statement. The results indicate that alexin is particularly susceptible to the action of ultraviolet light, and that the time required for complete destruction varies with the thickness of the layer exposed and the serum dilution. More recently the ground has been gone over again by Bovie,⁶ Brooks,⁷ and Sellards⁸ in studying the action of tropical sunlight and results obtained which are essentially in accord with the earlier work.

It has also been noted that the action of ultraviolet light may be inhibited by the addition to the photosensitive substance of blood serum, egg white⁹ and various other protein substances, or when the rays are first passed through a glass instead of a quartz vessel. This

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¹ Ledoux-Lebard: *Annal. l'Inst. Past.*, 1902, 16, p. 587; Raab, O.: *Ztschr. f. Biol.*, 1900, 39, p. 524; Raab, O.: *Ztschr. f. Biol.*, 1902, 44, p. 16; von Tappeiner, H., and Jodlbauer, A.: *München. med. Wehnschr.*, 1904, 51, p. 737 and 1139; von Tappeiner, H., and Jodlbauer, A.: *Deutsch. Arch. f. klin. Med.*, 1904, 80, p. 427; Lichtwitz, L.: *München. med. Wehnschr.*, 1904, 51, p. 1589; Pfeiffer, H.: *Wien klin. Wehnschr.*, 1905, 18, pp. 221 and 328; Sacharoff, G., and Sachs, H.: *München. med. Wehnschr.*, 1905, 52, p. 297; Fleischmann, P.: *München. med. Wehnschr.*, 1905, 52, p. 693; Hasselbalch, K. A.: *Biochem. Ztschr.*, 1909, 19, p. 435.

² *Arch. f. Hyg.*, 1905, 54, p. 53.

³ *Biochem. Ztschr.*, 1906, 1, p. 425.

⁴ *Compt. rend. Soc. de biol.*, 1910, 68, p. 393.

⁵ *Ztschr. f. Immunitätsforsch. u. exp. Therap.*, 1913, 19, p. 1.

⁶ *Jour. Med. Research*, 1918, 38, p. 335.

⁷ *Jour. Med. Research*, 1918, 38, p. 345.

⁸ *Jour. Med. Research*, 1918, 38, p. 293.

⁹ Henri, V., and Wurmser, R.: *Compt. rend. Soc. de biol.*, 1912, 73, p. 319.

protective action is due to the absorption of the ultraviolet rays by the protecting agent. Soret¹⁰ has shown that most proteins exhibit an absorption band in the ultraviolet end of the spectrum and that solutions of tyrosin exhibit this phenomenon most markedly. Kober¹¹ carried out spectrographic investigations on various amino-acids and split products of protein hydrolysis, and found that with the exception of the aromatic amino-acids, tyrosin and phenylalanin, the amino-acids, like the aliphatic acids, esters and certain other compounds investigated by Bielecki and Henri,¹² show no specific absorption. In the instance of the two mentioned aromatic amino-acids, the absorption is very marked and leads one to believe that the protective action afforded by certain proteins is due to their content of these amino-acids.

Harris and Hoyt,¹³ working in this laboratory, have confirmed these general results by studying the protection afforded to paramecia and certain bacteria by tyrosin and several other substances against the toxic action of ultraviolet light. It was this work that led to the present investigation which has for its object a more detailed study of aromatic substances as protecting agents against ultraviolet light. To measure this action we have chosen to work with alexin and the sensitizer for sheep cells, components of the well known hemolytic system, since these substances are easily measured, are very susceptible to the action of ultraviolet light, and exhibit moreover a difference in susceptibility toward these rays as well as toward heat. By determining the amount of either alexin or sensitizer to just cause complete hemolysis of the sheep cells, the amount of destruction due to exposure to the light as well as the protection afforded by various substances can be easily determined.

As a source of ultraviolet light we employed a Cooper-Hewitt—type Z—quartz mercury arc lamp, the tube being about 12 cm. above the solution to be tested for protective power. The latter was measured into quartz beakers mounted on black cardboard so that only the light passing through this solution could act on the serum contained in a shallow glass dish packed in ice. In the experiments on alexin, pooled guinea-pig serums were used in a dilution of 1:10 and sensitizer standardized in terms of alexin (0.1 cc), two units of the latter being used for the hemolytic system. In carrying out experiments with sensitizer, two serums were used, designated as A (1:9,500) and B (1:2,000) and the dilutions kept constant as originally standardized against alexin.

¹⁰ Arch. d. Sc. phys. et nat., Geneva, 1878, p. 322, and 1883, p. 194.

¹¹ Jour. Biol. Chem., 1915, 22, p. 433.

¹² Compt. rend. Acad. d. sc., 1912, 155, pp. 456, 1617; 1913, 156, pp. 550, 884, 1860; 157, p. 372; Berichte, 1912, 45, p. 2819; 1913, 46, p. 1304. See also, Baly, E. C. C., and Desch, C. H.: Jour. Chem. Soc. London, 1904, 85, p. 1029.

¹³ Sc., N. S., 1917, 46, p. 318; Univ. Cal. Pub. Path., 1919, 2, p. 245.

Table 1 shows the time required for destruction of alexin and sensitizer, absence of hemolysis indicating destruction. It will be noted that the unit of sensitizer is more easily destroyed than the unit of alexin, although a longer time is required for the lower (serum B) than for the higher dilution (serum A). The unit of sensitizer is, however, the same in both cases, and it is therefore probable that the protection afforded to B is due to the greater concentration of serum

TABLE 1

(A) SHOWING TIME NECESSARY TO DESTROY ALEXIN AND RABBIT VS. SHEEP CELL SENSITIZER BY EXPOSURE TO ULTRAVIOLET LIGHT

Substance Exposed	Time of Exposure, Minutes	Result Degree of hemolysis is indicated by +, ++, +++. Inhibition of hemolysis indicates destruction of alexin or sensitizer. 0.2 cc each of alexin and sensitizer used in the hemolytic system.				
		0.0 cc	0.2 cc	0.3 cc	0.4 cc	0.5 cc
Alexin 1:10.....	2	—	+++	+++	+++	+++
	4	—	—	—	+	++
	6	—	—	—	—	—
Sensitizer "A" 1:9,500.....	1	—	+++	+++	+++	+++
	2	—	—	—	++	+++
	3	—	—	—	—	—
Sensitizer "B" 1:2,000.....	3	—	—	+	++	++
	4	—	—	—	—	+
	5	—	—	—	—	—
Sensitizer "B" 1:10..... After exposure diluted 1:2,000 for hemolytic system	30	—	—	+	++	++
	45	—	—	—	+	++
	60	—	—	—	—	—

(B) PROTECTION AFFORDED TO ALEXIN AND SENSITIZER BY THE HOMOLOGOUS SERUM (INACTIVATED)

Protecting Substance	Time of Exposure, Minutes	Result				
		0.0 cc	0.2 cc	0.3 cc	0.4 cc	0.5 cc
Alexin 1:10.....	30	—	+	++	+++	+++
	45	—	—	+	++	++
Sensitizer "B" 1:10.....	30	—	—	+	++	++
	45	—	—	—	+	++

proteins. While the unit of sensitizer as compared with that of alexin is apparently more readily destroyed, yet alexin is actually more susceptible to the action of ultraviolet light than sensitizer. The apparent differences are due to dilution. When sensitizer in equivalent dilution (1:10) is exposed to ultraviolet rays 1 hour is required for complete destruction of the unit, while for alexin complete destruction takes place in about 5 minutes. The comparison between alexin and sensi-

tizer in equivalent dilutions is only approximate, since the time for destruction depends on the concentration of serum proteins which in the instance of the rabbit¹⁴ and the guinea-pig¹⁵ is different. Measured in terms of protective action the serums are not markedly different, as shown in the second part of table 1. The protective action of alexin and sensitizer in equivalent dilutions (both having been inactivated at 56 C.) for the homologous immune body was determined. Although alexin has been destroyed, the serum still possesses marked protective action, or in other words, ability to absorb ultraviolet rays.

TABLE 2
SHOWING PROTECTION OF ALEXIN BY VARIOUS SUBSTANCES WHEN EXPOSED TO THE ACTION OF ULTRAVIOLET LIGHT

Substance and Concentration	Solvent	Time of Exposure, Minutes	Result Degree of hemolysis is indicated by +, ++, +++. Inhibition of hemolysis indicates destruction of alexin. 0.2 c c alexin = 2 units.				
			0.0 c c	0.2 c c	0.3 c c	0.4 c c	0.5 c c
Glycocoll M/10.....	N/10 KOH	15	—	—	—	—	++
Alanin M/10.....	H ₂ O	15	—	—	—	—	+
Taurin M/10.....	N/10 KOH	15	—	—	—	—	—
Cystin M/20.....	N/10 HCl	15	—	—	+	+	++
Tyrosin M/20.....	N/10 KOH	90	+	++	+++	+++	+++
Phenylalanin M/20.....	N/40 NH ₄ OH	90	—	+++	+++	+++	+++
Phenylglycocoll M/20.....	N/20 KOH	90	—	++	++	+++	+++
Sulphanilic acid M/10.....	H ₂ O	90	—	+++	+++	+++	+++
Casein 1%.....	N/10 KOH	90	—	+++	+++	+++	+++
Gelatin 1%.....	H ₂ O	15	—	—	—	—	—
Witte's peptone 1%.....	H ₂ O	90	—	+++	+++	+++	+++
Protamin (salmin) sulphate 1%..	H ₂ O	30	—	++	++	+++	+++
		45	—	+	+	++	++
Amino benzoic acid M/10.....	H ₂ O	90	—	+++	+++	+++	+++
Sodium benzoate M/10.....	H ₂ O	90	—	+++	+++	+++	+++
Benzoic acid M/10.....	H ₂ O	90	—	+++	+++	+++	+++
Anilin 1%.....	H ₂ O	90	—	+++	+++	+++	+++

We next measured the protective power of various amino-acids, proteins, and certain substances of the aromatic series, the results being summarized in tables 2 and 3. The time of exposure as given is exclusive of the time necessary for destruction of immune body when the solvent alone is used as protecting agent. Proper controls were run to insure uniformity of conditions and destruction of immune body without protecting solution. It will be noted that alanin, glycocoll, taurin, and gelatin have little or no protective action against the destructive action of ultraviolet light. Marked protection is, however, shown by the aromatic amino-acids, tyrosin and phenylalanin and also

¹⁴ Woolsey, J. H.: Jour. Biol. Chem., 1913, 14, p. 433.

¹⁵ Wells, C. E.: Jour. Biol. Chem., 1913, 15, p. 37.

by various substances of the aromatic series. Casein and Witte's peptone both protect. It was expected that protamin (salmin) sulphate, due to its almost complete lack of aromatic amino-acids, would show no protective action; however, on testing our preparation, a positive test for tyrosin was obtained. This was true also for the preparation of cystin used. The results also show that the benzol ring is the determinative factor and not the groups attached to the ring. Thus anilin, benzoic acid, and sulphanilic acid protect equally well. The protection afforded by casein and the absence of any protective action shown by gelatin is due to the difference of aromatic amino-acid content, gelatin lacking these.

TABLE 3
SHOWING PROTECTION OF RABBIT VS. SHEEP CELL SENSITIZER BY VARIOUS SUBSTANCES WHEN EXPOSED TO THE ACTION OF ULTRAVIOLET LIGHT

Substance and Concentration	Solvent	Time of Exposure, Minutes	Result Degree of hemolysis is indicated by +, ++, +++. Inhibition of hemolysis indicates destruction of alexin. 0.2 cc alexin = 2 units.				
			0.0 cc	0.2 cc	0.3 cc	0.4 cc	0.5 cc
Glycocoll M/10.....	N/10 KOH	15	—	—	—	—	+
Alanin M/10.....	H ₂ O	15	—	—	—	—	—
Taurin N/10.....	N/10 KOH	15	—	—	—	+	+
Cystin M/20.....	N/10 HCl	30	—	—	+	+	++
Tyrosin M/20.....	N/10 KOH	90	—	+++	+++	+++	+++
Phenylalanin M/20.....	N/40 NH ₄ OH	90	—	+++	+++	+++	+++
Phenylglycocoll M/20.....	N/20 KOH	60	—	++	+++	+++	+++
Sulphanilic acid M/10.....	H ₂ O	90	—	++	+++	+++	+++
Casein 1%.....	N/10 KOH	90	—	+++	+++	+++	+++
Gelatin 1%.....	H ₂ O	15	—	+	+	++	+++
		30	—	—	—	—	—
Witte's peptone 1%.....	H ₂ O	90	—	++	++	+++	+++
Protamin (salmin) sulphate 1%..	H ₂ O	30	—	++	+++	+++	+++
		45	—	—	+	++	++
Amino benzoic acid M/10.....	H ₂ O	90	—	+++	+++	+++	+++
Sodium benzoate M/10.....	H ₂ O	90	—	+++	+++	+++	+++
Benzoic acid M/10.....	H ₂ O	90	—	+++	+++	+++	+++
Anilin 1%.....	H ₂ O	90	—	+++	+++	+++	+++

Note: In the first three determinations sensitizer "A" (1:9,500) was used. In the others sensitizer "B" (1:2,000) was used.

It is a fundamental concept in photochemistry that action by light cannot take place without absorption. Since absorption in proteins is largely due to the content of aromatic amino-acids it would seem to follow that in the case of substances such as the immune bodies, destruction is due to the content of aromatic amino-acids, unless the action be secondary as in the instance of leucin noted by Harris and Hoyt.¹³ It is possible that the difference in susceptibility shown by alexin and sensitizer is due to a difference in aromatic amino acid content.

SUMMARY

Although the unit of sensitizer is more easily destroyed by exposure to ultraviolet light than the unit of alexin, the difference is not real, since the concentration of serum proteins is enormously different. In dilutions more nearly comparable alexin is more quickly destroyed than sensitizer.

The aromatic amino-acids, tyrosin and phenylalanin, proteins containing these amino acids and certain substances belonging to the aromatic series show marked protective action for alexin and sensitizer against the action of ultraviolet rays.

Since destruction cannot take place without absorption it is possible that the difference in susceptibility shown by alexin and sensitizer toward ultraviolet light is due to a difference in aromatic amino-acid content.

THE CAUSE OF ABORTION IN MARES

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Studies of the etiology of abortion in mares have been made from time to time and reports from different localities assign as the probable cause an organism of the paratyphoid group. The work now presented was begun to determine whether there could be any relationship between abortions occurring among mares and an outbreak of influenza which was prevalent at this time on the farm. No such relationship could be established, but an organism has been isolated which is closely related if not identical with the one described by Meyer, Good and others. So far as known, this is the first report of the isolation of this organism in the west. Smith and Kilbourne¹ in 1893 isolated an organism resembling *B. suis* from an aborting mare in Pennsylvania. In 1897 Lignieres described an organism of the hog cholera group in an outbreak of epizootic abortion in mares in France and South America. In 1912 Good,² in Kentucky, isolated from the placenta and uterus of aborting mares and from the internal organs of aborted foals an organism of the intermediate subgroup of the colon-typhoid group, with which he produced abortion in animals including mares. For this organism he proposes the name *B. abortivo-equinus*. Van Heelsbergen³ and de Jong⁴ report the occurrence of abortion in mares in Holland, and the isolation of an organism resembling *B. paratyphosus* B. With this organism they succeeded in producing abortion in small animals and also in the mare and cow. Meyer showed that the agent of epizootic abortion of mares in Pennsylvania is a bacillus of the paratyphoid-enteritidis group and proposed the name of *B. abortus equi*.

Dec. 24, 1916, two mares were brought to the clinic, one (1088 A) having aborted during the night and the other (1088 B) showing restlessness and

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¹ Bureau of Animal Industry, Bulletin 3, 1893, p. 53.

² Ky. Agr. Exp. Station, Bulletin 165, 1912; Proc. U. S. Live Stock Sanitary Association, 1911, p. 114; Jour. Infect. Dis., 1913, 13, p. 53.

³ Centralbl. f. Bakteriologie, I, O., 1913, 72, p. 38; Ztschr. f. Infektionskrankh. der Haustiere, 1914, 16, p. 195.

⁴ Centralbl. f. Bakteriologie, I, O., 1912, 67, p. 148.

premonitory symptoms of abortion. Examination of the latter revealed a foal in anterior presentation with forelimbs retained by flexion at the elbow. The limbs were extended and traction exerted and delivery accomplished. The mares were isolated and all precautions taken against a spread of infection. The mares were from a winter pasture in which 8 others had been confined during the late fall and early winter. They had been fed cane with grain twice daily. Dec. 25 a third mare (1089) aborted in the same pasture; Dec. 27 four others; on Dec. 28 one other, and on Jan. 1 another. The following table shows stages of pregnancy:

TABLE 1
STAGES OF PREGNANCY IN MARES

Case	Bred	Aborted	Stage of Pregnancy
1,088A	May 15, 1916	Dec. 24, 1917	223 days
1,088B	May 17, 1916	Dec. 24, 1917	221 days
1,089	July 14, 1916	Dec. 25, 1917	164 days
1,091	July 1, 1916	Dec. 27, 1917	179 days
1,092	June 3, 1916	Dec. 27, 1917	207 days
1,093	Unknown	Dec. 27, 1917	
1,094	May 27, 1916	Dec. 27, 1917	214 days
1,095	May 27, 1916	Dec. 28, 1917	215 days
1,096	May 25, 1916	Jan. 1, 1917	221 days

Necropsy of the fetus, mare 1088 A, revealed the following: The fetal membranes were of a dark brownish-red color, rich in blood vessels and highly injected. The chorion and umbilical cord edematous and much thickened by serous infiltration. The skin normal, but subcutaneous tissue slightly infiltrated with a serous fluid. Over the surface of the placenta and the umbilical vessels was a fibrous exudate with papillary elevations of a grayish-white color, a condition observed in the membranes of all of the aborted foals, as well as in the fetuses of other species of animals aborted by injection of cultures from this fetus. Meyer and Boerner⁵ report a similar appearance of the chorion and amnion in one or more of the cases which came under their observation. The organism isolated was studied with results as follows:

Morphology.—The organism from the aborted fetus is a short, thick, ovoid rod with ends rounded, and a tendency to bipolar staining in the smears. The size varies from 0.2 to 0.5 mikrons by 0.5 to 1.5 mikrons. No uniformity of arrangement is observed, the organism usually lying singly, occasionally in pairs and rarely in short chains. It stains well with the ordinary stains and is gram-negative. In a hanging drop it is actively motile.

Isolation and Culture.—Isolation can be made directly from the fetal blood stream and the internal organs by plating or direct inoculation on plain agar, Endo agar or Drigalski medium.

Agar Streak.—After from 12 to 24 hours a grayish-white, glistening, abundant growth appears. After from 24 to 48 hours the growth becomes membranous and wrinkled and extends over the water of condensation as a dry appearing, brittle film. The water of condensation becomes turbid. Observations of old cultures show that the membranous condition persists.

Agar Plates.—Small, round, slightly elevated, glistening colonies, later showing a finely granular structure develop rapidly. These become dry, membranous and tough, and entire colonies may be moved along the surface of the agar

⁵ Jour. Med. Research, 1913, 29, p. 325.

with the platinum needle. The colonies in thickly sowed cultures remain pin point in size. If well separated they attain a size up to 4 or 5 mm. They remain discrete as a rule.

Gelatin.—Stab cultures are filiform. The surface growth is rapid and abundant, no liquefaction after 12 days' incubation.

Plain Broth.—After 24 hours the medium becomes uniformly turbid. After 48 hours a pellicle begins to develop which later settles to the bottom.

Milk.—After 10 days milk shows no coagulation, but changes to a yellow color. Reaction to litmus is alkaline.

Barsiekow 1 (Litmus-nutrose-dextrose).—Coagulation with marked acid production after 24 hours.

Barsiekow 2 (Litmus-nutrose-lactose).—This medium remains normal.

Hetsch (Litmus-nutrose-mannite).—The medium after 24 hours is milky. After 48 hours coagulation of the casein occurs, the color changes from rose to light pink and 40 to 60% of gas is formed after 72 hours.

Löffler 1.—Turbidity and gas formation, manifested by a greenish froth on the surface of the medium occur after 24 hours. Turbidity increases and after 48 hours a yellowish green precipitate of nutrose casein forms and gradually settles along the sides and at the bottom of the tube. A gradual clearing of the fluid at the upper part of the tube occurs.

Löffler 2.—For 24 hours the medium is unchanged. After 48 hours the medium loses its green color, rapidly changing from a dirty yellow to gray, and finally is colorless.

Litmus Whey.—After 24 hours the medium is dark red, changing to dark rose after 48 hours, to normal color after 72 hours, and to dark blue after 192 hours.

Neutral Red.—In 24 hours the medium begins to decolorize, gas formation marked by bubbles occurs and the fluorescence develops. Decolorization begins at the bottom, and after 192 hours only a narrow zone of red is left at the top.

Orcein Agar.—No change in this medium is noted.

Dextrose Broth.—Evolution of gas is rapid and in 72 hours from 48-50% of gas is present. The reaction is slightly acid.

Lactose Broth.—Growth is luxuriant in this medium but no gas is produced.

Dunham's Peptone.—No indol is produced. Pellicle formation is not so marked as on plain bouillon.

Drigalski Medium.—Colonies are blue.

Endo Agar.—White, disk-shaped colonies resembling those on plain agar develop after 18-24 hours.

Inoculation Experiments.—The culture used was isolated from the umbilical vein of the fetus of mare 1088 A, Dec. 24, 1916. Rabbit 150 was bred March 12, 1916; on April 2, two loopfuls (4 mg.) of a 24-hour agar culture in 2 c.c salt solution were injected intraperitoneally. April 4 mother rabbit aborted 3 dead fetuses and was killed; the point of injection was congested, the peritoneum slightly congested and covered with a purulent exudate. The external surface of the uterus was congested and hemorrhagic, internally slightly congested and covered with a thin weblike fibrinous exudate studded with granular elevations of a grayish-white color. The kidneys were slightly congested. The spleen was covered with thin fibrinous exudate.

The smears from peritoneal fluid, blood, uterine exudate, stained with methylene blue, all showed the characteristic organism. Pure cultures were obtained on agar; cultures were obtained also from an aborted fetus.

Mare 1.—This mare was bred July 4, 1916; on April 5, 1917, an intravenous injection of two loopfuls (4 mg.) of a 24-hour agar culture of the organism isolated from the uterus of the rabbit on April 4 was made. The mare aborted during the night of April 16. No premonitory symptoms were observed except the relaxation of the sacro-sciatic ligament, first noticeable on April 9, and a slight vaginal discharge on the evening of April 16. There were no noticeable disturbances of the temperature from April 4 to 24.

The fetal membranes were intact and the fetus was dead when discovered. Smears from the blood of the umbilical vein, the liver, the intestines, spleen, lungs and heart all showed organisms. The changes were as follows: Umbilical vessels covered with a fibrinous exudate on which were numerous papillary elevations of the color and appearance as in the uterus of rabbit 150. Maternal placenta covered with a slimy exudate on the internal surface; liver, slightly congested, a few hemorrhages; intestines, normal; lungs and heart, a few small hemorrhages; thymus, congested; spleen, congested and hemorrhagic. From the fetus cultures of the organism were obtained from the organs and blood.

Sow 1.—This was a cholera immune sow in about the twelfth week of pregnancy. May 5 she was injected intravenously with 2 loopfuls (4 mg.) of 24-hour agar culture in 2 cc of salt solution of the organism from the fetus of the aborted rabbit. On May 7 she aborted one pig, followed within a few hours by four others. The first pig showed the skin everywhere hemorrhagic; the peritoneal cavity contained a large quantity of serous fluid in which were the characteristic organisms; the layers of the peritoneum were hemorrhagic; the spleen was congested, and in the other organs there were many minute hemorrhages.

On the fetal placenta the characteristic granules seen in other placenta of aborted animals were present. The organism was recovered from this pig.

Guinea-Pig 192.—Inoculated intraperitoneally, Oct. 18, 1917, with 1 loopful of a 24-hour agar culture of bacillus from fetus of mare in 3 cc of salt solution. Since isolation, a period of 6 months, the organism had been transferred a number of times, being carried as a stock culture but had not been passed through any animals. Oct. 22, an edematous swelling appeared at the point of injection; the next day the animal had 3 dead young, partly haired, the skin of which showed a few hemorrhages; the subcutis and the thymus gland were congested; other organs apparently normal. Inoculations on agar from the blood, liver, and spleen were negative. The mother pig was killed a few hours after the abortion. A hard infiltrated area was present around the point of inoculation; slight inflammation of peritoneum in region of the uterus; inguinal glands on the side of inoculation enlarged; uterine walls thick, congested, lining deeply injected; liver mottled, brown; spleen showed numerous abscesses; other organs normal. The characteristic gram-negative bacillus was present in the blood, spleen, liver and uterus.

Agglutination tests by other investigators using as antigen organisms that are apparently the same as the one under consideration have shown that normal horse serum agglutinates in dilutions of from 1:40 to 1:300; my observations indicate that the lower titer is the commoner.

The agglutinating titers of serum from mares that have aborted from natural infection, as recorded by Meyer, may show a variation of from 1:1,200 to 1:2,500. DeJong⁴ records 1:1,000 as the highest titer of serum from mares that had aborted from a natural infection. My own observations show generally a lower titer (Table 2).

TABLE 2
AGGLUTINATION TESTS

Antigen	Titer		
	Mare 1,092 7 Days after Abortion	Mare 1,091 10 Days after Abortion	Mare 1,088A 101 Days after Abortion
Fetus 1,088A.....	640	320	320
Kentucky (Piatt).....	1,280	1,280	630
Kentucky (Sis).....	1,280	640	1,280
Pennsylvania (Reichel).....	640	320	320

The experimentally infected mare that aborted gave titers as follows:

Before abortion, Pennsylvania (Reichel).....	1:80
Three days after abortion, culture from fetus of mare 1088 A.....	1:1,280
Three days after abortion, Kentucky (Piatt)	1:1,280
Three days after abortion, Kentucky (Sis)	1:1,280
Three days after abortion, culture from own fetus.....	1:1,280
Three days after abortion, Pennsylvania (Reichel)	1:640
Eight days after abortion, culture from fetus of mare 1088 A.....	1:1,280
Eight days after abortion, culture from own fetus	1:640
Eight days after abortion, Pennsylvania (Reichel)	1:640

Serum from the sow drawn eight days after abortion showed an agglutinating titer of 1:2,500 for the strain of organism inoculated as compared with a titer of 1:20 of normal pig serum for the same organism.

Agglutination tests with highly immune serum from rabbits systematically immunized against the organism causing abortion present interesting evidence of a close relationship between this organism and organisms of the intermediate sub-group, Table 3.

TABLE 3
AGGLUTINATION TESTS WITH IMMUNE RABBIT SERUM

Antigen	Titer		
	Serum of Rabbit Immunized with Bacillus from Fetus, Mare 1,088A	Serum of Rabbit Immunized with Organism from Fetus, Mare 1	Serum of Rabbit Immunized with Kentucky (Piatt) Strain
Culture from fetus 1,088A.....	5,120	10,240	2,560
Kentucky (Piatt).....	5,120	10,240	2,560
Pennsylvania (Reichel).....	2,560	10,240	2,560
Culture, Mare 1.....	2,560	5,120	2,560
B. paratyphosus B.....	640	640	40
B. enteritidis.....	160	160	80
B. suispestifer.....	160	160	160
B. paratyphosus A.....	0	0	0
B. coli.....	0	0	0
B. paracoli.....	0	0	0
B. typhoses.....	0	0	0

Agglutination reactions using serum of rabbits immune to various organisms of the typhoid-enteritidis group against the different strains of *B. abortivo-equinus* gave results (table 4) that indicate relationships.

TABLE 4
AGGLUTINATION TESTS WITH TYPHOID-ENTERITIDIS SERUM (RABBIT)

Antigen	Titer				
	Antipara- coli Serum	Antityphi- murium Serum	Antienter- itidis Serum	Autipara- typhosus B Serum	Antipara- typhosus A Serum
<i>B. paracoli</i>	1,280	—	—	—	—
Fetus, Mare 1.....	0	320	320	160	320
Pennsylvania (Reichel)....	0	640	320	160	640
Kentucky (Piatt).....	0	320	160	160	640
Kentucky (Sis).....	0	640	320	160	640
<i>B. typhimurium</i>	—	1,280	—	—	—
<i>B. enteritidis</i>	—	—	2,560	—	—
<i>B. paratyphosus B</i>	—	—	—	5,120	—
<i>B. paratyphosus A</i>	—	—	—	—	5,120

Meyer and Boerner⁵ obtained these results with the organism isolated by them and serum from animals immune to members of the paratyphoid group: *B. paratyphosus A*, 1:20; *B. paratyphosus B*, 1:100; *B. enteritidis* 1:80. They also found that three paratyphoid strains were agglutinated by serum from rabbits immune to *B. abortivo-equinus* in dilutions from 1:20 to 1:100. *B. enteritidis* was agglutinated by the same serum in dilutions somewhat higher than the paratyphoid strains. A *suipestifer* strain was agglutinated at 1:600. They conclude from their results that the organism can be separated serologically from the main representatives of the paratyphoid-enteritidis group.

Lautenbach⁶ concludes on the basis of agglutination tests that the organism must be classed in the group of the hog cholera bacilli, and that it stands nearest *B. paratyphosus A*.

De Jong⁴ found that serum from two mares which had aborted agglutinated the organism found by him in dilutions as high as 1:1,000, while normal serum agglutinated only up to 1:300. A mare injected by him aborted on the 11th day and the organism recovered from the placenta was agglutinated by the serum from this animal in dilution of 1:1,000. A cow injected at the same time aborted in 15 days, and the organism recovered from the placenta was agglutinated by the serum of the cow in dilution of 1:600, while the titer of normal cow serum was 1:100. A mare fed a culture aborted and the bacillus was

⁶ Centralbl. f. Bakteriöl., I, O., 1913, 71, p. 349.

found in the fetus. The serum of this animal agglutinated the organism in dilution of 1:1,500 while normal horse serum gave a titer of 1:300. His conclusion is that the organism from aborted foals belongs to the paratyphosus *B. enteritidis* group, and that it is agglutinated in much higher dilution by the serum from aborted mares than by normal serum.

Good,² with serum from guinea-pigs immune to various members of the paratyphoid organisms, obtained little or no agglutination of the organism isolated by him from aborting mares.

Van Heelsbergen,³ using the serum of an artificially immunized mare with an agglutinating titer of 1:10,000, found that three different strains of *B. suipestifer* were agglutinated in dilutions no higher than 1:100. Three strains of *B. enteritidis* were not agglutinated above 1:300. Five strains of paratyphosus *B.* were agglutinated in dilutions of from 1:100 to 1:400, while *B. typhi-murium* was agglutinated in dilution of 1:2,400. His conclusion is that this behavior differentiates the bacillus not only from the swine pest organism, but also from the other different paratyphoid *B.-enteritidis* bacteria. He holds that up to the present time no single paratyphoid *B.-enteritidis* bacillus has been found which is agglutinated by a high dilution of the immune abortion serum that he prepared in the same manner as the specific strain, so that as a tentative test agglutination is the best method for the identification of the abortion bacillus. The only one of the paratyphoid *B.-enteritidis* group which is agglutinated in high dilution by abortion serum is *B. typhi-murium* of Loeffler, which was agglutinated in dilution of 1:2,400.

My results with agglutination tests more nearly correspond to those of Van Heelsbergen than to those of other investigators.

SUMMARY

From an outbreak of abortion in Iowa there was isolated an organism of the paratyphoid-enteritidis group, which in cultural and morphologic characters, and in serologic reactions is apparently the same as the organism studied by Good,² Meyer,⁵ and others, and variously named by them *B. abortivo-equinus*, *B. abortus-equi*, etc. The dry, brittle, membranous growth on slanted agar which was observed by these investigators was present in the cultures isolated in this outbreak, and this peculiarity is of great value in identification of the organism.

With this organism abortion was produced in the rabbit, guinea-pig, sow and mare by intraperitoneal or intravenous injection of minute doses in 2, 6, 2, and 11 days, respectively. Feeding and intravaginal introduction of the organism did not result in abortion in any animals thus treated.

The organism is agglutinated by immune serum for the organisms of paratyphoid-enteritidis group in fairly high dilutions. Serum of rabbits immune to this organism also agglutinate *B. suipestifer*, *B. enteritidis* (Gaertner) and *B. paratyphosus* A and B in dilutions high enough to indicate a close serologic relationship to these organisms.

THE BACTERIOLOGIC ANALYSIS OF THE FECAL FLORA OF CHILDREN

WITH NOTES ON THE CHANGES PRODUCED BY A CARBOHYDRATE DIET

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Certain extremely characteristic symptoms in children from 3 months to 8 years of age have for many years attracted the attention of one of us. These symptoms could well be explained by the assumption of an existing abnormal intestinal flora, so that we undertook a systematic study of these metabolic disorders of suspected intestinal origin by careful quantitative and qualitative bacteriologic stool examinations. The purposes of these tests were twofold: first, to verify and support the contention of the clinician that a putrefactive flora is associated with a definite syndrome in which bacteriologic data may possibly be of diagnostic value; secondly, to establish, by means of a standard technic, certain indexes by which the various types of fecal floras could be readily recognized, whereby the transforming influence of certain foodstuffs could be controlled.

Unfortunately, a complete method of analysis for infants' stools, based on the recent knowledge of gastro-intestinal bacteriology, was not available. We have, therefore, in the last year adopted a set of procedures which has been of great service in the study of the above mentioned intestinal disorders, and these will be detailed in this communication.

The technical procedures employed today in the study of the fecal flora are the outcome of a definite evolutionary development of the field of intestinal bacteriology. It would be of interest to review these historical facts, but for the sake of brevity we refer in this connection to the monographic presentations of the subject in the publications of Schmidt,¹ Sittler,² Escherich,³ Moro,⁴ Kendall,⁵ Torrey⁶ and others.

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¹ Die Fäzes der Menschen, 1910, p. 308.

² Die Wichtigsten Bakterientypen des Säuglingsdarmes, etc., 1909; Centralbl. f. Bakteriologie, 1908, 47, pp. 14 and 145.

³ Die Darmbakterien des Säuglings, 1886.

⁴ Jahrb. f. Kinderheilk., 1905, 61, pp. 687, and 870.

⁵ Bacteriology, General, Pathological, Intestinal, 1916, p. 579-600; Jour. Biol. Chem., 1909, 6, p. 499.

⁶ Jour. Infect. Dis., 1915, 16, p. 72-108.

MATERIAL AND METHODS

As already stated, it was our aim to establish by means of certain culture mediums and by microscopic examinations a standard by which the biologic activities of the fecal flora of children as a whole could be recognized. A total of over 250 specimens of diseased, and 12 samples of perfectly healthy children have been examined. In a series of cases repeated bacteriologic analyses were made to control the influence of diet on the improvement of the clinical symptoms. The stool specimens were all obtained from cases which exhibited the symptoms of intestinal intoxication described in another publication, and were under the constant supervision of one of us (L. P.). Stool specimens of children treated for surgical diseases, in the Children's Department of the University of California Hospital, served as controls; these patients did not suffer from intestinal disturbances and received a liberal mixed diet.⁷

METHODS OF EXAMINATION

The technic employed in this study was chosen after a careful critical experimental comparison of the methods suggested by Kerr, MacNeal and Latzer⁸ and those of Torrey. The methods described by the latter proved to be exceedingly valuable and were used in preference to the others. The various steps of the procedures finally chosen are presented in outline form in the paragraphs which follow. The selection of the cultural methods is by no means considered as final; in fact, we have added several improvements since we collected the data on which this paper is based. On the other hand, we hope that a description of a dependable technic will stimulate pediatricists to apply the same to the study of intestinal disorders of infants.

Collection of Stool Specimens.—Each stool was passed directly on a sterile piece of gauze and placed inside of a sterilized quart jar. Attempts were made to obtain specimens from the early morning movement. The samples were cultured in the next two or three hours after collection, immediately on their receipt in the laboratory. During the course of preparation for the analysis, the dilutions, etc., they were kept on ice.

TECHNIC FOR THE EXAMINATION OF A STOOL SPECIMEN

1. *Preparation of the "Stock Dilution":*

- (1) Weighing: weigh 500 mg. of stool on a sterile watch-glass.
- (2) Emulsifying: wash thoroughly into a sterile mortar with 10 c.c. of sterile salt solution; rub with pestle until a homogeneous emulsion, free from visible clumps, is obtained.
- (3) Dilution: transfer to a small Erlenmeyer flask and dilute with salt solution to a volume of 50 c.c. Each c.c. of this "stock solution" represents an equivalent of 10 mg. of fecal material. Fluid stools are emulsified in salt solution and standardized to the same density as one of the known weighed preparations.

⁷ The diet consisted of:

(a) Three-fourths formula:

Whole milk	600 c.c.
Water	200 c.c.
Dextro maltose 0.5 per cent. solution.....	24 c.c.
Barley water	11 c.c.

(b) One-half formula:

Whole milk	600 c.c.
Water	600 c.c.
Dextri maltose 0.7 per cent. solution.....	59 c.c.

⁸ Jour. Infect. Dis., 1909, 6, pp. 123 and 571.

2. Microscopic Examination and Differential Count:

- (1) Prepare smear from the "stock dilution" and stain by Gram's method.⁹
- (2) With the aid of a squared field ocular make a direct count of at least two fields of the following:
 - (a) Percentages of gram-negative organisms.
 - (b) Percentages of gram-positive organisms.
 - (c) Percentages of gram-positive rods.
 - (d) Percentages of gram-positive cocci.

3. Plating Procedures:

- (1) "Dally" one standard loop of stock dilution over the surface of one or two carefully dried Endo plates (Robinson's and Rettger's modification).
- (2) Sugar-free veal or beef liver-infusion-Difco-peptone-agar plates; reaction p_H 7.0-7.2.
 - (a) Prepare from the "stock dilution" the following dilutions: 1:1,000, 1:10,000, 1:100,000.
 - (b) With 1 cc of the dilutions 1:10,000 and 1:100,000 prepare pour-plates.
 - (c) Count plates after 24 hours aerobic incubation at 37 C.
- (3) Lactose agar plates (sugar-free-veal-infusion peptone—1 per cent. lactose agar).
 - (a) Prepare pour-plates using 1 cc of the dilutions 1:10,000 and 1:100,000.
 - (b) Count plates after 48 hours anaerobic incubation at 37 C.
- (4) Spore plate:
 - (a) Heat in a Pasteur pipet a portion of the stock dilution at 80 C. for 10 minutes.
 - (b) Make a pour-plate of lactose agar using one half of the heated suspension. Save the remainder for inoculation of a fermentation tube.
 - (c) Incubate aerobically for 48 hours at 37 C.

4. Seeding of the Fermentation Tubes:

- (1) 0.5 cc lots of the "stock dilution" are each seeded into the following tubes and incubated aerobically at 37 C. for 24 hours:
 - (a) 1 per cent. glucose sugar-free-veal-peptone broth fermentation tube.
 - (b) 1 per cent. lactose sugar-free-veal-peptone broth fermentation tube.
 - (c) 1 per cent. saccharose sugar-free-veal-peptone broth fermentation tube.
 - (d) Bromcresol-purple-milk-fermentation tube.¹⁰
- (2) Heated material from procedure 3, part 4, is inoculated into a fermentation tube of milk to which sterile defibrinated blood has been previously added.
 - (a) Incubate anaerobically at 37 C. for 48 hours.

5. Inoculation with Undiluted Stool:

- (1) One loopful of the undiluted fecal specimen is inoculated into the following mediums:

⁹ Sterling's or carbol methylviolet 6B or BN one minute, Gram's iodine solution—one minute, decolorized with acetone-alcohol (1:3) and counterstain with dilute carbolfuchsin.

¹⁰ Clark and Lubs: Jour. Agric. Res., 1917, 10, p. 105-111.

- (a) Gelatin stab tube melting point 28 C.¹¹
 - (b) Loeffler's serum slant medium.
 - (c) 1 per cent. lactose-peptone-ox-bile.
 - (2) Incubate (b) and (c) aerobically at 37 C. from 24 to 48 hours.
 - (3) Incubate (a) aerobically at 22 C. from 24 to 48 hours.
6. *Acetic Acid Glucose Broth Tubes:*
- (1) 0.5 cc of the "stock dilution" is inoculated in the following tubes:
 - (a) N/5 acetic acid veal-infusion—1 per cent. glucose-peptone-broth tubes.
 - (b) N/10 acetic acid veal-infusion—1 per cent. glucose-peptone-broth tubes.
 - (c) N/20 acetic acid veal-infusion—1 per cent. glucose-peptone-broth tubes.

For anaerobic cultures the plates are placed in a Novy or museum jar, from which the air is exhausted and the vacuum replaced by hydrogen. The results noted in the various culture mediums are recorded over a time interval of from 24 to 72 hours; the growth in the fermentation tubes is expressed in percentage of gas and by symbols designating the degree of turbidity. Liquefaction and digestion of gelatin and Loeffler's serum are tabulated as follows:

+ slight; ++ moderate; +++ rapid and heavy growth.

THE SIGNIFICANCE AND THE INTERPRETATION OF THE TESTS

The various culture mediums just described were chosen for the analyses of children's stools, because as a whole they produced a fairly true picture of the character of the stool flora and changes therein could be detected readily. For practical purposes it is reasonable to assume that the fecal flora represents the mirror picture of the flora of the lower portion of the intestinal tract; for accurate scientific work on the flora of the digestive tube, more extensive studies on necropsies of children are necessary before the findings and deductions derived on laboratory animals can be accepted as identical with those assumed in human beings. (See Sittler, 24, page 15.)

We distinguish three types of intestinal floras: The fermentative or saccharolytic; the facultative or normal; and the putrefactive or proteolytic type. Each of these is characterized by a certain definite group of bacteria which on culture mediums hold true to species and to type, and their biochemical activities produce certain end results which enable the bacteriologist to recognize the type of flora.

The obligate flora of the children's stool is unlike that of an adult; the cultural results of an infant's stool vary from the findings in adults that have been recorded by some of the writers mentioned in the introduction. These differences therefore need a detailed discussion which can well be incorporated in a consideration of the purpose of each bacteriologic test.

¹¹ For method of preparation see: Forster: *Centralbl. f. Bakteriol.*, 1897, 22, p. 341.

(1). *Microscopic Examination and Differential Count of the Direct Smears Made from the Feces and Stained by Gram's Method.*—Little need be said concerning the results obtained in the examination of the smears prepared from the "stock dilutions." In our experience we found that cultural procedures gave more reliable information with regard to the bacterial flora of the digestive tube. This conclusion is quite in accord with the statements made by Kerr, Latzer and MacNeal. Even the differential count can be very misleading and we hope that this abbreviated, antiquated form of stool analysis will ultimately be discarded as one of the routine procedures in the physician's office. On the other hand, a gram-stained stool smear is a considerable advance over nothing. It is not uncommon to find the pediatricist preparing slides with fat and starch stains, but he fails to investigate, even microscopically, the flora of the suspended fecal specimen.

(2). *Fermentation Tubes.*—Herter and Kendall¹² have both advocated the use of fermentation tubes containing sugar broths as important aids in a stool examination. The fact that these tubes offer an environment with varying oxygen tensions enables certain bacteria, usually present in the feces in very small numbers, to develop vigorously. According to the workers mentioned in the foregoing, the bacterial growth in a fermentation tube represents closely the viable microorganisms present in the stool. The volume of gas produced is, according to Kendall, of some diagnostic value. Under normal conditions of the digestive tube, closely similar amounts of gas are formed in the glucose, lactose and saccharose tubes. He also found that in adult cases suffering from intestinal disorders the percentage of gas usually increased above normal. On the other hand, in fermentative stools in which the aciduric organisms predominated, gas production was diminished. These general conclusions have not been confirmed through the studies of MacNeal, Latzer and Kerr, and Torrey. The amount of gas produced varied in the stool specimens of several normal individuals repeatedly examined by the first mentioned writers.

Smears prepared from the sediments of the different fermentation tubes and stained by Gram's method gave some insight into the occurrence of the various fecal bacteria. It may be said, however, that the relative distribution of the various organisms present was never clearly indicated inasmuch as the carbohydrate substances influence one or the other type to its advantage over the obligate flora. For

¹² Jour. Biol. Chem., 1909-10, 7, p. 203.

example, the saccharose fermentation tube always favored the development of streptococci and even when the presence of *B. bifidus* was otherwise demonstrated, this organism was suppressed and entirely absent in this particular medium. And again, the same stool specimen inoculated in lactose tubes would show a predominance of *B. bifidus* and the acidophilic bacilli with a suppression of the streptococci to a noteworthy degree. The lactose fermentation tube was therefore particularly valuable because it demonstrated the main flora of the infants' stool; the three characteristic pleomorphic forms of *B. bifidus*, so ably described by Herter in his book, were always readily recognized.

As a whole, the percentage of gas production was not so characteristic as it has been described for fecal specimens of adults. In interpreting this difference we offer the following explanation: An excess of lactic and acetic acid evolved by *B. bifidus* reduces the H-ion concentration of the medium to such a degree that the growth of the ordinary gas producing bacteria, like *B. coli* and *B. lactis aerogenes*, is partially or completely suppressed, and therefore no production of CO_2 takes place. In our experience this absence or low percentage of gas in the glucose and saccharose tubes is very characteristic for normal infant stools. In the lactose tube, however, the extensive growth of *B. bifidus*, as is to be expected, produced a certain percentage of gas which was always higher than in the other two carbohydrate tubes employed. The growth of streptococci and enterococci caused intensive turbidity with little or no gas production in the saccharose fermentation tubes. In our experience this phenomenon was particularly noticeable when dealing with highly putrefactive stools.

In four examinations after 48 hours' incubation the open arm of the fermentation tubes turned to a brilliant green when kept at room temperature and exposed to light, denoting the presence of *B. pyocyaneus*. Sugar tubes seeded with fermentative stool specimens frequently developed a thick pellicle consisting of an extensive growth of yeasts. These tubes also liberated the odor characteristic of these organisms.

Lactose-peptone-oxbile medium enriches the organisms of the *B. coli* group and the aerobic and anaerobic spore bearers. Again, the gas production was not characteristic, but the smears from the sediments frequently enabled us to make an early diagnosis of the nature of the stool. Briefly, the microscopic findings of the various sediments may

be as follows: in a putrefactive, proteolyzing flora the smears show few aciduric organisms, numerous *B. coli*, gram-positive spore-bearing rods, and streptococci; in a fermentative flora aciduric organisms predominate, and very few *B. coli* are noted.

The percentage of gas collecting in the closed arms was always higher than in the above discussed plain broth-carbohydrate tubes. This may in part be the result of a heavier seeding or the outcome of a restriction of the aciduric organisms which, as we have explained above, depresses the growth of the gas producers. Sometimes the percentage of gas offered suggestions for a possible diagnosis of the fecal flora, namely, when more than 60 per cent. of gas had collected in the closed arm. In these instances the specimen invariably contained a large number of *B. welchii*, as could be checked by the stormy fermentation which took place in the blood milk tubes. The maximum gas production as a rule was only recorded after 48 hours; the bacterial lag was probably affected by the alkaline reaction of the bile, which inhibited to a certain degree the biochemical activities of the inoculated bacteria.

The milk-fermentation tubes containing bromcresol purple¹⁰ as a delicate indicator have proven in our hands to be of extreme value. Irrespective of the type of flora, whether putrefactive or fermentative, the reaction of the inoculated and incubated tubes was acid; the aciduric and *B. coli* organisms are naturally responsible for this result. Therefore, it is not the reaction but its constant occurrence that gives the character of the curd diagnostic importance. Stormy fermentation with maximum whey production was only recorded when *B. welchii* was present. A soft, yellowish curd with few gas bubbles and little or no whey was found to be produced by a fecal specimen containing primarily proteolytic bacteria. Such a curd on further incubation showed progressive peptonization and intensification of the yellow color. Organisms found in fermentative stools produce another very important reaction in the milk: The curd is firm, massive, with considerable whey; the closed arm may contain from 20 to 30 per cent. of gas. Frequently lenticular gas bubbles are seen in the otherwise smooth curd. Peptonization or changes in the color never take place, even after prolonged incubation. The descriptions of the two kinds of curds apply naturally only to the absolutely fixed types of a putrefactive or a fermentative flora. We have frequently noted, however,

less significant changes and have found it difficult to interpret these "pseudo reactions" when examined independently of the other mediums employed.

(3). *Loeffler's Serum Medium Slant and Gelatin Stab Culture.* — These two mediums supply a protein rich substratum and therefore favor organisms commonly encountered in putrefactive stool specimens. The degree of liquefaction and digestion of the respective mediums may well serve as a criterion for the number of viable putrefactive bacteria. In our experience the Loeffler's tube is an excellent diagnostic aid in stool analyses. Incubated for from 24 to 72 hours, the coagulated serum may undergo the following changes: either small lenticular and irregular depressions, so-called "biting in," accompanied by slight discoloration or complete digestion with intensive blackening of the dissolved serum and a strong putrid odor always proved the existence of a putrefactive stool sample. The organisms most frequently found in such peptonized tubes were bacteria of the *B. coli* group, streptococci and gram-positive sporulating rods. Detailed systematic studies still in progress have convinced us that the aerobic and anaerobic gram-positive spore-bearing rods in all probability constitute some of the constant elements of a putrefactive stool specimen of children. Slight growth, absence of liquefaction or changes in color and odor were associated with fecal bacteria of strictly fermentative specimens.

Gelatin stab cultures showed liquefaction of varying degrees, depending entirely on the source of the sample examined. Pronounced putrefactive feces caused complete liquefaction in 72 hours. In our series of observations three types were commonly recorded: (a) liquefaction along the needle tract with arborescent spreading into the depth of the medium; (b) liquefaction beginning about one-half to one inch below the surface of the gelatin column with little extension into the depth. This type indicates the presence of organisms with a delicately adjusted requirement for oxygen; (c) infundibuliform surface liquefaction progressing downward, with amber-brownish discoloration of the dissolved medium, suggests aerobic gram-positive spore bearers.

Until we have completed our study of the predominant organisms found in a putrefactive stool, it appears to us premature to interpret these types of liquefaction. It is not unlikely that certain clinical intoxications of gastro-intestinal origin are associated with a definite

group of proteolytic organisms; and therefore the types of gelatin liquefactions just described may have considerable diagnostic value.

The bacteria commonly found in a fermentative stool specimen changed the heavily seeded gelatin column and the Loeffler's serum slant in a few instances only. The microscopic examination of these cases demonstrated a large number of gram-positive spore bearers. Most of the specimens which gave this irregular reaction were derived from cases recently placed on a carbohydrate diet.

(4). *Plate Counts on Sugar-Free Agar Aerobically, and on Lactose-Agar Anaerobically.*—The counts obtained and calculated represent the number of viable micro-organisms per one milligram of fecal matter. The sugar-free, as well as the lactose-agar plates supplied some information concerning the relative distribution of the various important stool bacteria. In sugar-free-agar plates the majority of the non-aciduric organisms develop, as for example: *B. coli*, streptococci, etc. On the other hand, the very important aciduric bacteria multiply only in a medium containing carbohydrates, particularly lactose, and in an environment with reduced oxygen tension. When all the growth enhancing factors are fulfilled, these bacteria thrive satisfactorily in colonies, and a plate count represents the true number of viable facultative aciduric organisms. The following criteria served as aids in the recognition of the aciduric bacteria, *B. bifidus* and *B. acidophilus*; namely, marked pleomorphism of the gram-positive, fine rods, lenticular gas bubbles surrounding the surface colonies, and pronounced acetic odor of the plates. *B. bifidus* as a rule forms an oval or round colony with peripheral gas bubbles, while *B. acidophilus* is characterized by a cottony, fluffy, tuftlike center colony which is frequently surrounded by a dense turbid ring of sister colonies, giving a peculiar clouding of the medium.

The interpretation of the various counts obtained on the lactose-agar plates was sometimes difficult. As is well known, infants' stools always contain *B. bifidus* and *B. acidophilus* even in the most marked putrefactive stools. Therefore, the mere presence of aciduric colonies on the plates (in the ratio of 1:1) was not a criterion of a fermentative character of the specimen. Only when the ratio of the aciduric organisms is in a proportion of 2:1, is the flora certainly fermentative.

(5). *Endo Plates.*—In our hands this medium proved to be invaluable because an early diagnosis of the nature of the fecal material could be given. This medium, properly inoculated, will supply, even

on casual examination, immediate striking information. Putrefactive stools produce a heavy growth consisting of a variety of different types of colonies. A large spreading surface-growth covering the entire plate surrounds small groups of *B. coli* and possibly streptococci. The reaction of the medium is usually strongly alkaline, a red discoloration being noted only at the edges of the agar disk. This type of plate also gives forth a sweetish, pungent, musty odor. Quite in contrast to these findings are those recorded when a fermentative stool specimen has been plated. Few colonies, invariably those of *B. coli*, with their typical deep red color spreading throughout the medium, produce a marked acid odor. Similar results are obtained on bromcresol purple plates.

(6). *Spore Bearing Bacteria in Lactose-Agar Plates and Milk-Blood Tubes*.—Lactose-agar plates poured with the heated “stock dilution” specimen demonstrate the presence of aerobic spore-bearing bacteria. As a rule, growth was only noticed after from 48 to 72 hours’ incubation. Gram-positive spore-bearing bacteria were regularly found in putrefactive stools; numerous and repeated examinations of stools recognized by other methods to be fermentative failed to demonstrate this group of organisms.

Fermentation tubes containing milk and, as an enrichment substance, blood, were always inoculated with the heated stool dilutions. Stormy fermentation with the findings of characteristic nonmotile gram-positive rods proved to be an indication of the presence of *B. welchii* or closely allied bacteria. In our studies we failed to find this organism in fermentative stools; putrefactive specimens, however, contained this bacterium frequently in large numbers. According to Herter,¹³ *B. welchii* is considered to be one of the most important factors of intestinal putrefaction, being responsible for the so-called “saccharobutyric type” of gastro-intestinal intoxication. We are not as yet prepared to regard the *B. welchii* as one of the main causative organisms of intestinal proteolysis, but we are impressed with the absence of this organism in stool specimens of children kept on a high calory carbohydrate diet.

(7). *Acetic Acid Glucose Broth Tubes*.—In strongly putrefactive stool specimens the absence of aciduric bacilli is regularly shown by sterile acetic acid tubes. Marked growth in N/10 and N/5 acetic acid broth is therefore significant for a fermentative fecal flora.

¹³ Bacterial Infections of the Digestive Tract, New York, 1907.

THE MAIN CHARACTERISTICS OF A NORMAL, A PUTREFACTIVE AND
A FERMENTATIVE STOOL

From the above description of the methods and the interpretation of the findings, it is quite apparent that the three main types of fecal floras observed in children readily can be diagnosed with a great deal of accuracy. We summarized the results of about 150 different stool specimens in table 1. The data therein recorded represent the basic criteria by which a stool should be diagnosed as normal or as putrefactive or as fermentative.

Before we enter into a discussion of the bacteriologic differences between a normal, a putrefactive and a fermentative fecal flora, it is appropriate to say a few words concerning the macroscopic appearance of the stool samples. As a rule, the color and consistency of the specimen does not suggest the type of flora present except in fermentative types. A light buff-colored, foamy, semiformal stool specimen with a sour odor and strongly acid reaction to litmus or other indicators, is very suggestive of a fermentative fecal flora. On the other hand, proteolytic or putrefactive floras have been noted in fecal samples that can be grouped in the following three types: (1) dry, formed, solid dark-brown stools; (2) light colored, moist, mucus-containing stools with considerable undigested food debris accompanied by an offensive, putrid odor; and (3) samples of dirty, greenish color of semiformal, coarse consistency. The stool specimens of normal children have no characteristic macroscopic appearance, and vary according to the diet.

Returning to the discussion of the cultural findings in the various types of feces, we note from the figures and readings given in table 1, that the differences between a fermentative and a putrefactive flora are sufficiently striking, and therefore need little emphasis. The same cannot be said when we analyze the data characteristic of a normal stool. A correlation of the findings in normal with those in pathologic stools appears, therefore, to be of interest.

It is noted from table 1 that the fecal floras of an artificially fed child not suffering from any intestinal disturbances or the symptoms of a gastro-intestinal intoxication, resemble those of a slightly putrefactive stool. This fact is explained by the repeatedly made observation that infants fed on cow's milk have in the digestive tube a flora which is represented by a greater variety of bacteria than one commonly encountered in nurslings. Furthermore, the aciduric types of bacteria are suppressed and therefore an environment is created which

TABLE 1

PROTOTYPE OF STOOL SPECIMENS EXAMINED

	Normal Stool (12 specimens examined):				Putrefactive Stool (90 specimens examined):				Fermentative Stool (25 specimens examined):			
	Diet: Bovine milk				Character of Stools: Rather soft; light in color; odor normal				Character of Stools: See text			
	Direct Count of Smear (average count of 12 specimens examined):				Direct Count of Smear (average count of 90 specimens examined):				Direct Count of Smear (average count of 25 specimens examined):			
	24 hours	48 hours	72 hours		24 hours	48 hours	72 hours		24 hours	48 hours	72 hours	
Gelatin stab 22 C.	+	+	++		+	++	+++		—	—	—	
Cresol-purple milk fermentation tube....	9% gas Acid reaction Clot shrunken Whey expression +	No softening of clot	Peptoniza- tion +		4% gas Acid reaction Clot very soft No whey ex- pression	Partial peptoniza- tion +	Partial peptoniza- tion ++		46% gas Acid reaction Clot very shrunken; Whey expression ++	No softening of clot	No	
Fermentation tubes:												
1% glucose broth.....	22.2%		21.3%		22.2%	
1% lactose broth.....	21.3%		23.2%		34.3%	
1% succharose broth.....	23.7%		9.9%		19.8%	
Acetic acid glucose broth N/5.....	++	++	++		+	+	+		++	++	++	
Acetic acid glucose broth N/10.....	++	++	++		++	++	++		++	++	++	
Acetic acid glucose broth N/20.....	++	++	++		++	++	++		++	++	++	
Lactose agar spore plate.....	No growth —	No growth —	No growth —		Growth +	Growth +	Growth +		No growth —	No growth —	No	
Loeffler's serum slant 37 C.	Digestion — Discolora- tion —	Digestion + Discolora- tion +	Digestion + Discolora- tion +		Digestion + Discolora- tion +	Digestion ++ Discolora- tion ++	Digestion ++ Discolora- tion ++		Digestion — Discolora- tion —	Digestion — Discolora- tion —	D	
Lactose agar plate anaerobic.....	564,000		784,400		621,850	
Sugar-free plate aerobic.....	647,000		628,450		451,240	
Lactose bile fermentation tube.....	37%	54%		31%	46%		55.5%	
Endo plate aerobic 37 C.	Normal plate; acid reaction; growth ++				Proteolytic plate; alkaline reaction				Saccharolytic plate; acid reaction; g			
Blood milk fermentation tube.....	Stormy fermentation				Stormy fermentation				No stormy fermentation			
anaerobic												

is most favorable for the numerous proteolytic bacteria of infants' stools resulting from the feeding of cow's milk. The existence of these conditions is well indicated by the figures of the number of colonies recorded in the anaerobic and aerobic plates. The anaerobic bacterial count is approximately the same in all three types of infant floras; the aerobic count, which is due to *B. coli*, cocci and possibly a variety of spore-bearing rods, is noticeably reduced in a fermentative stool. In a normal stool this count is high and corresponds to the one noted in proteolytic specimens. The predominance of these aerobic

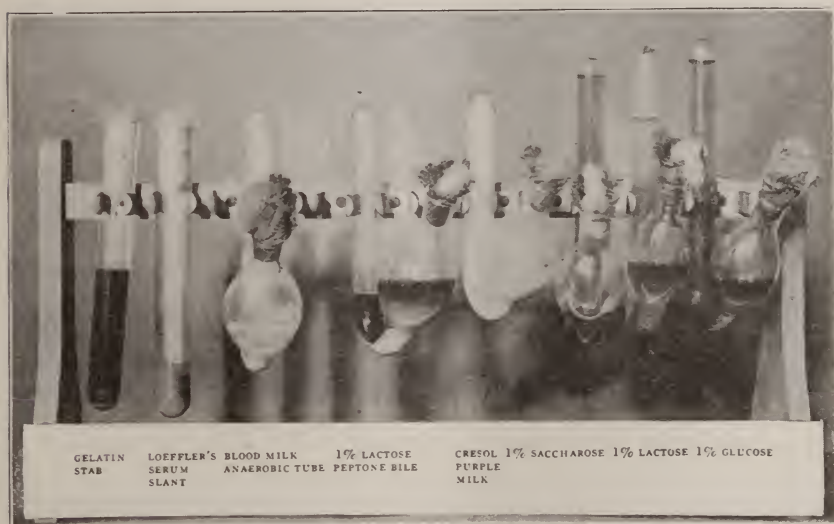


Fig. 1.—Main cultural reactions of a putrefactive fecal specimen.

organisms which have been introduced into the intestinal tract by the cows' milk, are the agents responsible for gelatin liquefaction; slight peptonization of the Loeffler's serum slant, and the partial softening of the milk curd.

Torrey¹⁴ has been able to show quite recently, through carefully conducted experiments, that milk and a high casein diet stimulate a vigorous growth of saprophytic streptococci and enterococci. We made similar observations; the smears from the Loeffler's serum tubes — seeded with stool specimens derived from children fed on cows' milk — regularly demonstrated a predominance of streptococci. Also

¹⁴ Jour. Med. Res., 1919, 39, p. 415.

Endo plates smeared with such stool emulsions revealed a majority of cocci which together with *B. coli* rendered the reaction of the plate distinctly acid.

In proceeding to the discussion of the putrefactive type of fecal flora, one is impressed with its similarity to the normal type of a cow's milk stool. There are only differences in degree, and it is sometimes exceedingly difficult to distinguish a semiputrefactive flora from a normal one. On the other hand, a putrefactive flora, which according to our conception may be responsible for clinical symptoms, is always

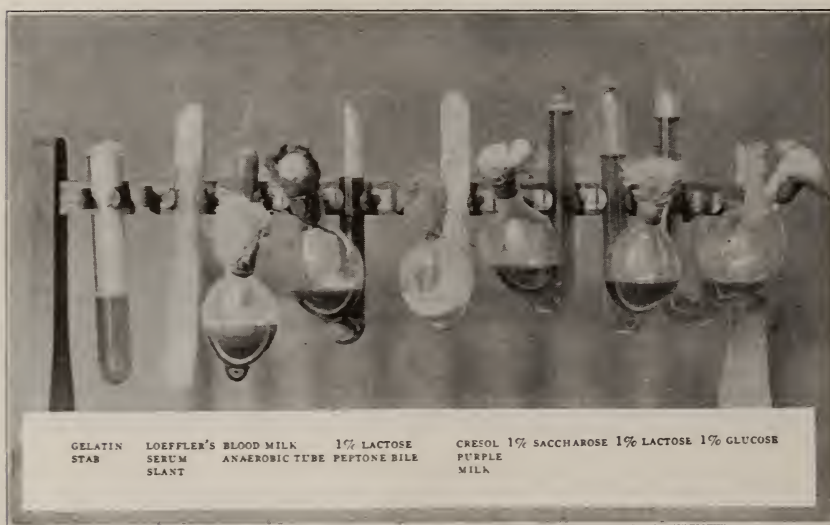


Fig. 2.—Main cultural reactions of a fermentative fecal specimen.

characterized by the following striking changes in the cultural tests: rapid liquefaction of the gelatin, the Loeffler's serum slant and the milk curd, as well as a strongly alkaline reaction of the Endo plates.

From a purely bacteriologic standpoint one is naturally interested in the question: Which bacterium or which group of organisms, or which "symbiotic complex" constitutes the poisonous elements of an obligate putrefactive flora? A number of chemists and experimental pathologists have repeatedly attempted to solve this complicated problem and several groups of organisms are thought to be responsible for the production of readily demonstrable toxic substances, like indol and phenol, in the intestinal content or the urine.

Herter¹³ considers the *B. coli* and *B. welchii*, perhaps certain strains of *B. proteus*, of *B. putrificus* and of "*B. malignant oedema*"(?) either alone or in combination, responsible for intestinal putrefaction. The recent studies of Berthelot¹⁵ and Rhein¹⁶ on *B. coli* phenologenes and those of Van Loghem¹⁷ and Groot¹⁸ on the *B. proteus* anindologenes contribute considerable weight to the conclusions of Herter.

It is not unlikely that these various types become acclimatized to the digestive tubes of certain infants, act as obligate parasites and by their activities produce a putrefactive fecal flora (Jehle and Pincherle¹⁹). Naturally, this contention can only be proved by a systematic analysis of a large number of such stools and by a careful chemical study of the cleavage product of the isolated gram-negative rods on specially devised culture mediums.

The importance of *B. welchii* as a proteolytic organism, although functionally saccharolytic, calls for a revision of the published interpretations of the vast number of carefully collected facts, in the light of our more recent conception of the toxigenic properties of this bacteria and our growing knowledge concerning the difficulties of studying obligate anaerobes.

From personal experimentation we agree with Tenbroeck²⁰ that *B. welchii* undoubtedly plays only a very subordinate rôle in summer diarrhea. Recent observations have shown, however, that this organism may be responsible for some of the grave symptoms of intoxication in infantile dysentery. It is generally known that the accompanying flora of infantile dysentery is usually strongly putrefactive, containing an abnormal number of *B. welchii* (alkaline reaction of the stool). Following the dicta of Kendall, the pediatricist who is not especially trained in intestinal bacteriology is inclined to treat such cases by a strict lactose and carbohydrate regimen. The sudden shift in diet will naturally suppress the growth of the *B. dysenteriae*, but will also supply an excess of fermentable carbohydrates to the existing *B. welchii* and the other gas-producing intestinal bacteria. In case the strict lactose diet is continued for more than from 24 to 48 hours, in our experience grave symptoms of gas bacillus intoxications with sudden death are not uncommon.

¹⁵ Ann. de l'Inst. Pasteur, 1918, 32, p. 17.

¹⁶ Biochem. Ztschr., 1917, 84, p. 246.

¹⁷ Ann. de l'Inst. Pasteur, 1918, 32, p. 295.

¹⁸ Ann. de l'Inst. Pasteur, 1918, 32, p. 2.

¹⁹ Wien. klin. Wchnschr., 1910, 23, p. 94.

²⁰ Bost. Med. and Surg. Jour., 1916, 174, p. 785.

How far this organism is responsible for a putrefactive flora we are not prepared to state definitely. As Ford, Blackfan and Batchelor²¹ have stated, the mere presence of anaerobic, gram-positive rods is not indicative of anaerobic putrefaction, nor are these rods of diagnostic value since they are found in a variety of clinical conditions. The observations of Wollstein that the stools of infants contain more spores of *B. welchii* when kept on a protein than on a carbohydrate diet is fully confirmed in our study of several hundred stool specimens. We wish, furthermore, to emphasize the fact that a high carbohydrate diet has a striking inhibitive influence on *B. welchii*. According to Kendall and Day²² and to the more recent work of Wolf and Harris,²³ *B. welchii* is fairly sensitive to a variety of acids, and cessation of growth occurs at P_H 4.82. It is therefore logical to produce an acid environment in the digestive tube of sufficient degree that multiplication of *B. welchii* is completely suppressed. The mere introduction of aciduric bacilli alone, as suggested by Kendall,²⁴ naturally will not bring about the desired result. In the absence of the proper pabulum of carbohydrates, the *B. acidophilus* can only lead a very limited intestinal existence, as has been emphasized repeatedly.

In only one or two of our patients with intestinal intoxication was the number of *B. welchii* spores so excessive that a sudden shift from a mixed diet to a pure carbohydrate one would have rendered the conditions for multiplication of this organism more favorable, and aggravated the clinical symptoms. The following principle was therefore applied: By means of starvation, if necessary by a cathartic and by feeding the child for from 24 to 48 hours on a strict protein diet, reduce the actual number of viable *B. welchii*, and then gradually substitute the pure proteid and buttermilk diet for one rich in carbohydrates. Progressively with the change of the putrefactive flora to a fermentative one, the number of spores decreases steadily until the methods we employed failed to demonstrate their presence. Again we offer this observation as a suggestion for further work on the rôle of the anaerobes in intestinal infections. In several instances we have isolated other anaerobes aside from *B. welchii*, and particularly in experimental work on dogs the frequent ubiquitous distribution of *B. bifermentans* and *B. sporogenes* along the large area of the digestive

²¹ Am. Jour. Dis. Child., 1917, 14, p. 354.

²² Boston Med. and Surg. Jour., 1912, 159, p. 754.

²³ Biochem. Jour., 1917, 11, p. 213-245.

²⁴ Am. Jour. Med. Sc., 1918, 156, p. 157.

tract emphasized the fact that not every gram-positive sporulating gas-producing anaerobe is really a *B. welchii*.

The constant presence of strongly proteolytic gram-positive spore-bearing aerobes in the putrefactive stools has impressed us with the possible importance of this group of bacteria. The types thus far identified correspond with those described by Batchelor.²⁵ Torrey¹⁴ considers these organisms of no significance from a biologic standpoint. We are not inclined to take such a radical view. It may be considered as having been proven through the studies of Ford, Blackfan and Batchelor²¹ that these aerobic spore bearers are mechanically introduced with certain types of food, protein milk, farina, etc., richly infected with such bacteria. Changes in diet may, however, favor the growth, and under certain conditions a direct implantation in symbiosis with other bacteria may produce a suitable environment and render these fortuitously present micro-organisms the contributors of harmful cleavage products. It is our impression that a systematic inquiry into the various micro-organisms thus far accused of being responsible for intestinal intoxication is not complete without a consideration of the spore-bearing gram-positive aerobes.

The same suggestion can also be applied to the conception that *B. putrificus* is perhaps the organism responsible for intestinal putrefaction. The frequent occurrence of this bacterium, which has thus far been studied very superficially in putrefying protein material (Tulloch²⁶) and also, for example, in pulp decay, is in many respects very suggestive. The description of two definite types of *B. putrificus* by Kligler²⁷ strongly emphasized the need of a more detailed study of this group of anaerobes. A possible adaptation of certain types to the intestines cannot be denied until further studies have been completed. And particularly the influence of aerobic bacteria on the growth of *B. putrificus* has to be considered. When viewed from this standpoint, the microbial associations of aerobic spore bearers in their relation to some of these anaerobes at least gain in importance as factors in a putrefactive fecal flora.

In turning to a discussion of the findings in a fermentative stool, some striking differences immediately attract our attention: complete absence of liquefaction of the gelatin and the Loeffler's serum slant, acid reaction of the Endo plate with a comparatively low count of the

²⁵ Jour. Bacteriol., 1919, 4, p. 23.

²⁶ Jour. Royal Army Med. Corps, 1917, 29, p. 631.

²⁷ Jour. of the Allied Dental Soc., 1915, 10, p. 321.

aerobic organisms. These cultural findings are the result of one established fact, namely, the predominance of aciduric bacilli, *B. bifidus* and *B. acidophilus*. An overwhelming presence of these organisms naturally has a depressing effect on the majority of saprophytic bacteria, which in turn produces an exceedingly simple type of flora. In contradistinction to the normal and putrefactive types of flora with a striking variety of bacterial species, the fermentative flora therefore consist of a few types only. The inhibitive effect of the acid metabolic split product of the aciduric organisms is particularly clearly shown in the disappearance of the fortuitously ingested streptococci and spore-bearing gram-positive rods. The latter statement applies not only to the aerobes, but to the anaerobes as well. The observations of Kendall explained that a high carbohydrate diet initiates through the metabolic activities of the aciduric organism such a low H-ion concentration of the intestinal content that *B. welchii*, for example, is unable to germinate. Therefore, it is not surprising that in our series of stool analyses stormy fermentation of the anaerobic milk tube was never observed. Clinical observation also supports our views that *B. welchii* and its supposedly harmful effects are more readily controlled by an eventually high carbohydrate than by a strict protein diet.

As is to be expected, not all of the stool specimens examined conform in their bacteriologic results to the three prototypes just discussed. Many reactions, sometimes rather puzzling, are obtained, and it is, therefore, considered necessary to present some of these results in table 2.

Two analyses of each of the three types are summarized. Fecal floras which differ from the recognized standards are designated as semiputrefactive and semifermentative, respectively. It is considered unnecessary to enter into a detailed discussion of their origin. We only desire to emphasize their occurrence and to impress the bacteriologist with the fact that only repeated bacteriologic examination of the feces by a uniform technic will sometimes establish the true nature of fecal flora.

A rapid and satisfactory diagnosis of the character of the fecal flora is sometimes possible by an abbreviated method. In cases of infantile dysentery the customary use of Endo or bromcresol-purple plates will, in conjunction with the isolation of the specific pathogenic dysentery bacilli, invariably suggest the character of the fecal flora. The detailed discussion given in previous paragraphs amply supports

TABLE 2

EXAMPLES OF PRONOUNCED AND MODIFIED TYPES OF FLORA

	Normal Stools			Putrefactive Stools			Fermentative Stools		
	Type, Normal 1	Type, Normal 2	Name, Normal 1	Pronounced Putrefactive	Name, Baby Cannon	Character of Stool; light in color; semi-formed; spongy	Character of Stool; formed; light in color	Name, Baby Lugori	Character of Stool; foamy; no color
	Name, Normal 8	Name, Normal 1	Character of Stool; Formed; color normal; odor bad	Name, Baby Cannon	Character of Stool; light in color; semi-formed; spongy	Name, Baby Lugori	Character of Stool; foamy; no color	Name, Swartz	Character of Stool; dark in color; dry
	Direct Count of Smeear: Percentage gram-negative organisms, 61.9% Percentage gram-positive organisms, 38.1% Percentage gram-positive rods, 82.6% Percentage gram-positive cocci, 27.9% Date, Aug. 5, 1918	Direct Count of Smeear: Percentage gram-negative organisms, 55.5% Percentage gram-positive organisms, 44.5% Percentage gram-positive rods, 53.6% Percentage gram-positive cocci, 46.4% Date, Aug. 3, 1918	Character of Stool; Formed; color normal; odor bad	Direct Count of Smeear: Percentage gram-negative organisms, 33.6% Percentage gram-positive organisms, 66.4% Percentage gram-positive rods, 42.6% Percentage gram-positive cocci, 57.4% Date, Sept. 19, 1918	Direct Count of Smeear: Percentage gram-negative organisms, 45.8% Percentage gram-positive organisms, 54.2% Percentage gram-positive rods, 63.7% Percentage gram-positive cocci, 36.3% Date, July 23, 1918	Direct Count of Smeear: Percentage gram-negative organisms, 15% Percentage gram-positive organisms, 85% Percentage gram-positive rods, 55% Percentage gram-positive cocci, 45% Date, Sept. 18, 1918	Character of Stool; foamy; no color	Name, Swartz	Character of Stool; dark in color; dry
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
	+	+	+	+	+	+	+	+	+
	Acid reaction 7% gas clot formed whey +	Acid reaction 12% gas clot shrunk whey ++	Acid reaction 21% 25% 33% 35%	Acid reaction 3% gas clot soft whey --	Acid reaction 10% gas clot semi-soft whey +	Acid reaction 46% 48% 31%	Acid reaction 15% 36% 5%	Acid reaction 40% gas clot firm whey ++	Acid reaction 15% 36% 5%
	21% 22% 25% 28%	21% 25% 33% 35%	21% 25% 33% 35%	22% 33% 28%	22% 33% 28%	22% 33% 28%	22% 33% 28%	22% 33% 28%	22% 33% 28%
	+	+	+	+	+	+	+	+	+
	No growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth
	Digestion +	Digestion +	Digestion +	Digestion +	Digestion +	Digestion +	Digestion +	Digestion +	Digestion +
	Discoloration +	Discoloration +	Discoloration +	Discoloration +	Discoloration +	Discoloration +	Discoloration +	Discoloration +	Discoloration +
	369,600	409,200	340,000	812,000	960,000	1,432,000	660,000	865,000	420,000
	45%	45%	45%	30%	30%	28%	40%	55%	77%
	Acid reaction; normal plate; growth ++	Acid reaction; normal plate; growth ++	Acid reaction; normal plate; growth ++	Alkaline reaction; proteolytic plate	Alkaline reaction; proteolytic plate	Alkaline reaction; semiprotoleolytic plate	Alkaline reaction; semiprotoleolytic plate	Acid reaction; succharolytic plate	Acid reaction; succharolytic plate
	No stormy fermentation	No stormy fermentation	No stormy fermentation	Stormy fermentation, B. welchii present	Stormy fermentation, B. welchii present	Stormy fermentation, B. welchii present	No stormy fermentation	No stormy fermentation	No stormy fermentation

this contention. For clinical use we have selected the gelatin stab tube, Loeffler's serum slant, cresol-purple milk with and without blood, 1 per cent. lactose peptone bile in fermentation tubes, and Endo plates. The results, which are characteristic for the various types of fecal floras, are self-explanatory and are shown in table 3.

REPEATED STOOL ANALYSES IN CHILDREN TREATED BY A STRICT
CARBOHYDRATE DIET

It can be considered an established fact that the microbial flora of the intestinal tube stands in direct relationship to the diet of the host. Ingestion of even large numbers of bacteria foreign to the digestive tract does not itself seem to displace the common intestinal types in normal individuals. The studies of Herter^{7, 8} and Kendall,²⁴ of Rettger and his pupils,^{28, 29} and of Torrey^{6, 14} demonstrate clearly this correlation between the types of bacteria found in the intestines and the clinical composition of the ingested food. The absence of carbohydrates in the diet produces a predominance of the proteolytic bacteria, which in turn may be responsible for putrefactive processes in the intestines, and by a continued absorption of the resulting toxic products are suspected by the clinician to be the main factors of some of the symptoms mentioned in the introduction.

The gratifying results obtained in the treatment of typhoid fever by a high carbohydrate diet, which through the painstaking studies of Torrey⁶ can in part be explained by the development of a more favorable flora for the patient, immediately suggested to the pediatricist the use of a similar diet for the treatment of certain intestinal intoxication in children.

Originally, it was thought possible to apply the procedures of Torrey, as outlined in his work on the influence of a carbohydrate diet on the intestinal flora of typhoid patients, to the problem of the intestinal disorders of infants. The conditions of a private practice unfortunately prevented a careful quantitative control of the various elements of the diet ingested and systematic regular stool analyses were not possible. And again, the amounts and proportions of the carbohydrates fed were frequently changed, making it difficult to determine which substance exerted the most beneficial effect. The data

²⁸ Jour. Bacteriol., 1917, 2, p. 47.

²⁹ Centralbl. f. Bakteriologie, I, 1914, 75, p. 219.

TABLE 3
ABBREVIATED FORM OF STOOL ANALYSIS

	Normal Stool			Putrefactive Stool			Fermentative Stool		
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
Gelatin tube	Liquefac- tion +	Liquefac- tion +	Liquefac- tion ++	Liquefac- tion ++	Liquefac- tion +++	Liquefac- tion +++	Liquefac- tion —	Liquefac- tion —	Liquefac- tion +
Loeffler's serum slant.....	Digestion and discolora- tion +	Digestion and discolora- tion +	Digestion and discolora- tion ++	Digestion and discolora- tion +	Digestion and discolora- tion +++	Digestion and discolora- tion +++	Digestion* and discolora- tion —	Digestion and discolora- tion —	Digestion and discolora- tion +
Cresol-purple milk tube.....	Acid reaction Gas + Whey expres- sion + Clot soft	Peptoniza- tion +	Peptoniza- tion +	Acid reaction Gas ± Whey expres- sion + Clot soft	Peptoniza- tion ++	Peptoniza- tion ++	Acid reaction* Gas ++ Whey expres- sion +++ Clot firm	Peptoniza- tion —	Peptoniza- tion —
1% lactose peptone bile.....	Gas production, 15 to 30% Smear of sediment: B. coli; gram-positive cocci; few gram-positive rods			Gas production, 10 to 25% Smear of sediment: B. coli; gram-positive rods many gram-positive rods			Gas production, 10 to 50% Smear of sediment: B. coli, few aciduric		
Endo plate	Reaction, acid Growth, ++ Odor, Acid Types of colonies, varied			Reaction, alkaline Growth, +++ Odor, pungent and offensive Types of colonies, Numerous			Reaction, acid Growth, + Odor, strongly acid Types of colonies, few		

* The interpretation of irregular reactions is found in paragraph (3) dealing with findings in Loeffler's serum medium slant and gelatin stab cultures (page 356).

TABLE 4

EFFECT OF A STRICT CARBOHYDRATE DIET ON PATIENTS WHOSE STOOLS HAVE BEEN DIAGNOSED PUTREACTIVE

Name of patient.....	Jean Adams				Semiformal; no odor; foamy; moist				Formed; no odor; moist; light in color			
	Semiformal; light; odor bad				Second examination, June 21 (22 days later)				Third examination, July 15 (24 days later)			
	First examination, May 29				48 hours				48 hours			
Character of stool.....	Type of Flora: Putrefactive				Type of Flora: Semi fermentative				Type of Flora: Fermentative			
Direct count of smear: Percentage gram-negative organisms.....	25.1% 74.9 %				11.3% 88.2% 71.5% 28.5%				8.0% 92.0% 87.4% 12.6%			
Percentage gram-positive organisms.....	54.1%				14% gas				Acid reaction			
Percentage gram-positive rods.....	45.9%				Clot firm				5% gas			
Percentage gram-positive cocci.....	Whely +				Clot firm				Clot firm			
Gelatin stab 22 C.	++				—				—			
Cresol-purple milk fermentation tube.....	Acid reaction				Acid reaction				Acid reaction			
1% glucose	6% gas				14% gas				5% gas			
1% lactose	Clot soft				No gas				2% No gas			
1% saccharose	Whely +				Clot firm				Clot firm			
Acetic acid glucose broth N 5.....	17% 12% 10%				5% 2% No gas				5% 2% No gas			
Acetic acid glucose broth N 10.....	++				++				++			
Acetic acid glucose broth N 20.....	++				++				++			
Lactose agar spore plate.....	No growth				No growth				No growth			
Loeffler's serum slant 37 C.	Digestion ++				Digestion ++				Digestion ++			
Lactose agar plate anaerobic.....	Discoloration ++				Discoloration ++				Discoloration ++			
Sugar-free agar plate aerobic.....	Digestion ++				Digestion ++				Digestion ++			
Lactose bile fermentation tube.....	Discoloration ++				Discoloration ++				Discoloration ++			
Endo plate aerobic 37 C.	No growth				No growth				No growth			
Blood milk fermentation tube anaerobic	4.3% gas				20% gas				90% gas			
	Alkaline reaction; proteolytic plate; growth ++				Acid reaction; saccharolytic plate; growth +				Acid reaction; saccharolytic plate; growth +			
	Stormy fermentation				Stormy fermentation				Stormy fermentation			
	Stormy fermentation				Stormy fermentation				Stormy fermentation			

TABLE 4—Continued
EFFECT OF A STRICT CARBOHYDRATE DIET ON PATIENTS WHOSE STOOLS HAVE BEEN DIAGNOSED PUTREFACTIVE

Name of patient.....	Richard Hargreaves									
Character of stool.....	Liquid; greenish; undigested food									
	First examination, July 18									
	24 hours	48 hours	72 hours	Type of Flora: Putrefactive						
	++	+++	+++							
	10% gas Clot soft Whey +	Slight softening of clot	Peptonization ++							
	</									

TABLE 4—Continued
EFFECT OF A STRICT CARBOHYDRATE DIET ON PATIENTS WHOSE STOOLS HAVE BEEN DIAGNOSED PUTREFACTIVE

Name of patient.....	Grace Holdkerman									
	Semiformed; odor very bad; moist; dark									
	First examination, June 29									
Character of stool.....	Type of Flora: Putrefactive					Type of Flora: Semiputrefactive				
Direct count of smear: Percentage gram-negative organisms... Percentage gram-positive organisms... Percentage gram-positive rods..... Percentage gram-positive cocci.....	42.6% 57.4% 61.2% 38.8%	48 hours	72 hours	48 hours	72 hours	32.8% 67.2% 81.5% 19.5%	48 hours	72 hours	32.8% 66.5% 76.2% 23.8%	Type of Flora: Semiputrefactive
Gelatin stab 22 C.	++	+++	+++	+++	+++	+	+	++	+	72 hours
Cresol-purple milk fermentation tube...	Acid reaction 90% gas Clot soft Whey —	Clot softening Peptoniza- tion +	Peptoniza- tion ++	No softening of clot	Peptoniza- tion ++	Acid reaction — gas Clot soft formed	No softening of clot	Peptoniza- tion ++	Acid reaction — gas Clot soft	Peptoniza- tion +
Fermentation tubes: 1% glucose 1% lactose 1% saccharose	24% 24% 5%	20% 19% 20%	23% 18% 7%
Acetic acid glucose broth N/5..... Acetic acid glucose broth N/10..... Acetic acid glucose broth N/20.....	++ ++ +++	+++ +++ +++	+++ +++ +++	+++ +++ +++	+++ +++ +++	++ +++ +++	++ +++ +++	++ +++ +++	++ +++ +++	++ +++ +++
Lactose agar spore plate.....	Growth +	Growth ++	Growth ++	Growth ++	Growth ++	Growth +	Growth +	Growth +	No growth	No growth
Loeffler's serum slant 37 C.	Digestion + Discolora- tion —	Digestion ++ Discolora- tion ++	Digestion ++ Discolora- tion ++	Digestion ++ Discolora- tion ++	Digestion ++ Discolora- tion ++	Digestion + Discolora- tion —	Digestion + Discolora- tion ++	Digestion ++ Discolora- tion ++	Digestion + Discolora- tion —	Digestion + Discolora- tion ++
Lactose agar plate anaerobic.....	267,000	1,410,000	1,140,000
Sugar-free agar plate aerobic.....	237,800	2,625,000	1,230,000
Lactose bile fermentation tube.....	27%	68%	57%
Endo plate aerobic 37 C.	Alkaline reac- tion; proteo- lytic plate; growth +++	Slightly alkali- line reaction; semiproteo- lytic plate; Growth ++	Slightly alkali- line reaction; semiproteo- lytic plate; Growth ++
Blood fermentation tube anaerobic.....	Stormy fer- mentation	No stormy fermentation	No stormy fermentation

Semiformed; light brown in color; odor

Third examination, Dec. 2 (2 months later)

TABLE 4—Continued

Name of patient.....		Frederick Roeding			
Character of stool.....		Liquid; dark brown; odor bad			
		First examination, July 18			
Direct count of smear:		48 hours	72 hours		
Percentage gram-negative organisms...	43.51%	++	+++	Type of Flora: Putrefactive	
Percentage gram-positive organisms...	56.49%				
Percentage gram-positive rods.....	44.40%				
Percentage gram-positive cocci.....	66.00%				
		24 hours	48 hours	72 hours	
Gelatin stab 22 C.	++		++	+++	
Oresol-purple milk fermentation tube...	Acid reaction	Softening of clot	Peptonization ++		
	10% gas	Peptonization +			
	Clot firm				
	Whey +				
	21 %
	1% glucose
	1% lactose
	1% succharose
Acetic acid glucose broth N/5.....	+	+	+	+	+
Acetic acid glucose broth N/10.....	++	++	++	++	++
Acetic acid glucose broth N/20.....	++	++	++	++	++
Lactose agar spore plate.....	No growth	Growth ++	Growth ++		
Loeffler's serum slant 37 C.	Digestion +	Digestion +	Digestion +		
	Discoloration —	Discoloration +	Discoloration +		
	555,000
Lactose agar plate anaerobic.....	400,600
Sugar-free agar plate aerobic.....	36 %
Lactose bile fermentation tube.....	Alkaline reaction; proteolytic plate; growth +++
Endo plate aerobic 37 C.	Stormy fermentation
Blood fermentation tube anaerobic.....					

to be presented indicate therefore only a further application of the methods discussed, but they offer certain suggestions of considerable practical value.

A total of twelve cases was examined clinically by one of us (L. P.); stool specimens were forwarded to the laboratory when the child was first seen by the clinician. Subsequent fecal specimens were only analyzed when improvement in the clinical picture was definitely manifest, or when the child showed an unexplainable relapse. Inasmuch as most of the cases were not systematically investigated, we present here, in tables 4, 5, 6 and 7 only the summarized bacteriologic findings of four cases. The technical procedures and the interpretation of the findings are the same as already outlined. The treatment offered these cases, aside from a high calory carbohydrate diet, consisted of mild stimulation of the intestinal mobility to ensure the carbohydrates reaching the big bowel. Details concerning this phase are presented in the clinical paper of this series.

From all of the rather casually collected data one fact preeminently stands out; namely, the fecal flora of a child which shows the syndrome (tired look, dark circles around the eyes, liquid or constipated stools, anorexia, cyanosis, stupor and semiconsciousness, and laxity, recently described by one of us — L. P.) is always strongly putrefactive and rich in a variety of proteolytic bacteria. The actual number of aciduric organisms is always low, particularly in case 3, shown in table 6. In our experience such cases present a very unfavorable "facultative" intestinal flora, and changes in diet accomplish only slowly the desired increase in the aciduric bacilli. When a carbohydrate diet is prescribed for such cases, but the execution of the regimen is rather lax, relapses are common and in every instance the bacteriologic examinations showed a return from a semifermentative flora to a strongly putrefactive one. On the other hand, a clinical improvement or even a complete cure from an apparently chronic intoxication by the use of a strict carbohydrate diet was, in our examinations, always strikingly indicated by a fermentative flora. The increase of aciduric bacilli over the number noted at the first examination was always very marked; in some cases this group of organisms outnumbered the other viable bacteria about 15 or 25 to 1. The cultural results on the anaerobic lactose agar plate, the acetic acid glucose broth tubes, and the direct counts presented in the tables give sufficient evidence to that effect. Therefore, the results thus far collected prove

that the fecal flora of a child's intestinal tract may be changed through a strict, liberal carbohydrate diet to such a degree that it reverts toward, or completely to, the fermentative non-gas-producing flora of the nursing.

In this connection it may be mentioned that individual differences play an important rôle. Torrey in dogs, and Hull and Rettger²⁹ in rat experiments observed that changes were more readily effected in some individuals than in others. In our experience clinical recovery from these intestinal intoxications can only be associated with a complete transformation of the putrefactive-proteolytic type of stool to a decidedly fermentative one. Such a result is usually only accomplished after a prolonged, strict carbohydrate diet regimen. The mere elimination of the obligate putrefactive organisms and a moderate development of the aciduric types is not sufficient. This particular fact explains in part the long time interval which as a rule was necessary to transform the initial strongly putrefactive flora. If, however, the initial flora was more favorable, semiputrefactive only in character, a change through a carbohydrate diet can be readily achieved in from 2 to 6 weeks.

It is generally stated from experiments on laboratory animals that a transformation of the fecal flora through carbohydrate diet may be accomplished in a few days. In our experience with children with an unfavorable initial flora from 10 to 40 days are, as a rule, required for this transformation. Aside from the individual differences of the initial flora of the patients already discussed, the number and variety of types of organisms and the length of the infants' intestinal tubes exceed those of many lower animals. It is therefore not surprising that the time element to produce a complete transformation must be taken into account from a therapeutic point of view. In fact, our observations on children are well supported by the data which Torrey has collected from the examinations of adult cases. Even in those patients with an initial favorable "facultative" intestinal flora, at least two weeks were required to increase through a carbohydrate diet the *B. acidophilus* to a level characteristic of an obligate fermentative flora.

As already stated, we were unable to make systematic examinations of the feces at regular time intervals. We therefore cannot say how soon the obligate putrefactive organisms were suppressed, after the strict regimen of a carbohydrate diet had been instituted. We know from the case histories that sometimes several weeks elapsed before clinical improvement was noticeable. Judging from this and from the

fact that improvement is not possible without a definite transformation to a predominant aciduric simplified flora, we also feel justified in concluding that a change in flora does not occur inside of a few days. The transformed flora persists as long as the special diet is continued. Only under a diet with animal proteins eliminated and with a predominant carbohydrate foundation are the results more than transitory.

Some observations also have shown that intercurrent and possibly focal infections like tonsillitis or rhinitis have an "inhibitive" influence on the normally progressing transformation of a harmful putrefactive flora. Tonsillectomy was associated, in a few instances, with a remarkably rapid change of flora, which, in spite of a strict carbohydrate diet enforced by feeding of *B. acidophilus* and other therapeutic measures, had remained stationary for several months. The various factors responsible for this influence can only be surmised until further studies have been completed, and are therefore reserved for another publication.

The main result of this study of selected cases under treatment is, in our experience, shown by the reliable information and the diagnostic value which detailed stool analyses offer to the clinician. The application of the procedures outlined to a variety of infants' diseases may furnish data in the future which, together with the clinical observations, will help to unravel the manifold factors of so-called gastro-intestinal intoxication in infancy.

In this connection the next most important question is, Which food-stuffs exert the strongest transforming influence on the intestinal flora? Most of the workers in this field of applied bacteriology agree that the feeding of such carbohydrate foods which on hydrolysis readily yield dextrose, as for example lactose, are most suitable. Torrey¹⁴ has shown that in adult typhoid fever cases kept on a regimen of 250 gm. and upward of lactose, there resulted a transformation of the ordinary type of flora to one strongly dominated by *B. acidophilus*. On the other hand, Sittler² demonstrated that the feeding of carbohydrates which on hydrolysis yield levulose (cane sugar) tend to bring about a mixed flora with an overwhelming number of *B. welchii*. Maltose, lactose and dextrose are the substances most suitable for the control of proteolytic organisms of the digestive tube. Experiments on children and dogs, with different foodstuffs, were in progress when quite recently our studies along these lines were materially enlightened by the excellent study of Torrey. By experiments on dogs he was able to show that lactose and dextrin added to the diet completely suppress

the proteolytic types of fecal bacteria, even including *B. coli* commonly found in the dog's intestinal tract. Starchy foods (white bread, potatoes and beans) also tended to effect a simplification of the intestinal flora.

Among the proteins responsible for intestinal putrefaction, milk casein exhibited the tendency far less markedly than did meat protein. Vegetable proteins also fail to offer encouragement to the growth of proteolytic organisms. We have confirmed these observations in a few feeding experiments on dogs and we await with great interest the application of this knowledge to the treatment of children showing intestinal intoxications. We have thus far gained the impression that in the children's digestive tube the effect of cow's milk proteins is almost, if not quite, as conducive to the growth of proteolytic bacteria as is meat protein in the dog's intestinal tract.

Children with an unfavorable initial flora respond very slowly and incompletely to the transforming influence of a carbohydrate diet, as already discussed in detail. In such cases it may be advisable to implant aciduric organisms by feeding pure culture or tablets of *B. acidophilus*. It is not unlikely that the application of the so-called "broma therapy" of Kendall, the intestinal implantation of the beneficial *B. acidophilus*, should be combined with the carbohydrate diet and made a routine procedure in all intestinal, or even in acute, infections of childhood (Kendall). In a few cases of infantile dysentery we have seen excellent results by the use of this principle. Until more cases have been studied bacteriologically, we offer the above considerations merely as suggestions to the clinician.

SUMMARY

This article contains the description and interpretation of a bacteriologic method applicable to the examination of fecal specimens of children apparently suffering from "intestinal intoxication." It is furthermore shown that a strongly putrefactive flora is associated with certain groups of intestinal disorders of infancy, and that clinical improvement is practically always accomplished by a strict carbohydrate diet. The progress in the transformation of the intestinal flora is readily controlled by the cultural tests described.³⁰

³⁰ In addition to the references given, the following may be of interest:

Moro: *Johr. f. Kinderh.*, 1905, 61, pp. 687 and 870.

Rettger and Horton: *Centralbl. f. Bakteriol.*, 1914, 73, p. 362.

Tissier: *Recherches sur le flore intestinale des nourrissons*, These de Paris, 1900.

Tissier: *Ann. de l'Inst. Pasteur*, 1905, 19, p. 109.

Wollstein: *Am. Jour. Child.*, 1912, 4, 279.

STUDIES IN EPIDEMIC ENCEPHALITIS (ENCEPHALITIS LETHARGICA)

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In a previous article¹ we presented the results of a number of inoculation experiments in monkeys, and showed that by intracerebral injections of an emulsion of brain tissue in salt solution from a fatal case of epidemic encephalitis we had succeeded in producing in monkeys lesions resembling closely those in man. In that article we described as failures secondary inoculations of brain from monkey to monkey. However, subsequent study of the brain of one of these monkeys (monkey 2) has revealed that we had been led astray by the presence of a gross hemorrhage, which we then interpreted as possibly traumatic. This inoculation we now know to have been successful, both by the presence of typical microscopic lesions at a distance from the hemorrhage, and also by successful inoculation of rabbits as now described.

Successful transmission to monkeys were recorded with the use of Berkefeld N filtrates of both nasopharyngeal washings and nasopharyngeal mucous membrane from encephalitis patients. A secondary inoculation in the monkey was obtained with the filtrate of the nasopharyngeal mucous membrane from a fatal case of epidemic encephalitis. We were unable to produce lesions with the filtrates of the nasopharyngeal mucous membrane from a case of cardiovascular disease.

In this article we present the results of further experiments on monkeys and of a series of experiments on rabbits.

MONKEYS

MONKEY 9 (*M. Cynomolgus*).—On April 25, 1919, the nasopharynx was painted with filtrate of nasopharyngeal mucous membrane from Wanemacher in 50% glycerin.

May 28: No effect to date.

MONKEY 10 (*M. Cynomolgus*).—On April 25, 1919, 2 cc of filtrate of nasopharyngeal mucous membrane from fatal case of epidemic encephalitis were injected subdurally.

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¹ New York Med. Jour., 1919, 109, p. 772.

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May 2: Monkey had recovered after a period in which she manifested typical lethargy, general malaise, elevated temperature, and ptosis of the left lid. She was practically moribund at one time. The monkey was allowed to recover for subsequent use in immunity experiments.

MONKEY 4 (M. Rhesus).—On March 22, 1919, 2 c.c. of filtrate of nasopharyngeal washing from patient, Gordon, with epidemic encephalitis who had recovered were injected subdurally over the left parietal fossa.

March 30: Paresis of both hind legs. The monkey was apathetic; huddled in the corner of the cage and did not respond to stimuli.

April 10: It had fully recovered after a severe illness lasting 10 days.

May 2: Same virulent filtrate of nasopharyngeal mucous membrane as in monkey 10 was injected as follows: 0.5 c.c. intracerebrally and 1.5 c.c. subdurally in the left parietal region, 2 c.c. subdurally in the right parietal region.

September 26: Perfectly normal to date.

MONKEY 17 (Lemur Catta).—On June 25, 1919, it was injected subdurally with 2 c.c. of filtrate of nasopharyngeal mucous membrane from (control) fatal surgical case.

September 26: No ill effects to date.

RABBITS (CHART 1)

Wherever possible, medium sized rabbits or hares were used to permit of intracranial inoculation by means of simple needle puncture, care being taken to pass just within the skull.

EXPERIMENT 1

RABBITS 3, 4, 7.—On April 25, 1919, they were injected intracranially with filtrate of the nasopharyngeal mucous membrane from a fatal case of epidemic encephalitis.

April 29: Rabbit 4 died. Examination revealed punctate hemorrhages on convexity of brain and intense general congestion; marked meningitis with infiltration of mononuclear leukocytes. Vessels in meninges show perivascular infiltration. There were hemorrhages in cortex, also vessels of cortex.

RABBITS 5, 6.—On April 29 they were injected intracranially with filtrate of brain of rabbit 4.

April 20: Rabbit 5 died. There were hemorrhages over convexity of brain and intense congestion; marked meningitis, mostly mononuclear leukocytes; slight perivascular infiltration; mononuclear infiltration of brain tissue; hemorrhages in cortex.

May 9: Rabbit 6 died. There was moderate congestion; also marked meningitis with mononuclear leukocytes and perivascular infiltration of vessels reaching into cortex from pia.

RABBITS 8, 9, 10.—May 2: The rabbits were injected intracranially with filtrate of brain of rabbit 5.

May 3: Rabbit 8 died. Examination revealed intense congestion; marked meningitis with mononuclear leukocytes mostly; foci of infiltration in cortex with mononuclear and polynuclear leukocytes; areas of intense perivascular infiltration with invasion into surrounding brain tissue.

May 3: Rabbit 9 died. Examination revealed intense congestion; meningitis with mononuclear leukocytes; intense perivascular infiltration of mono-

nuclear leukocytes; present also in basilar ganglions. There were areas of mononuclear infiltration with surrounding zone of necrosis; edema of the brain and marked engorgement of blood vessels.

RABBITS 13 AND 14.—On May 9, 1919, they were injected intracranially with filtrate of brain of rabbit 8.

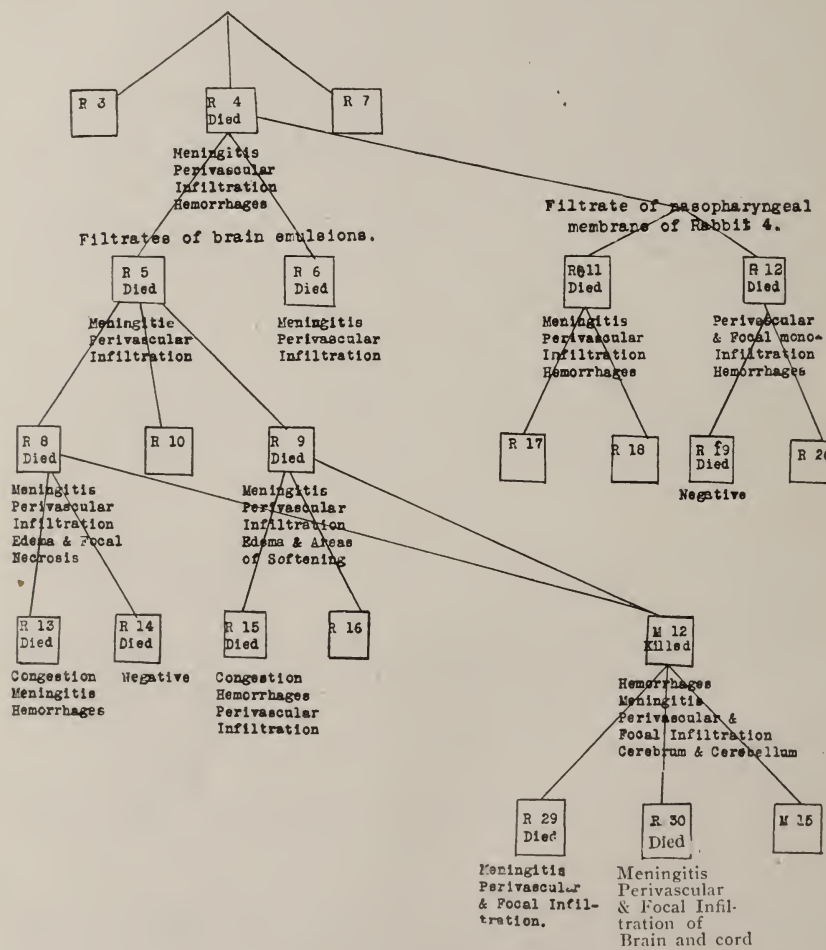


Fig. 1.—Inoculations of Rabbits.

May 15: Rabbit 13 died. Examination revealed intense congestion including congestion of vessels of the pia; one small perivascular hemorrhage; one area of hemorrhage and necrosis.

May 25: Rabbit 14 died. There were intense congestion; small hemorrhages; minute hemorrhages in basilar ganglions; numerous punctate hemorrhages in cerebrum, perivascular infiltration; edema; moderate mononuclear leukocytic meningitis.

RABBITS 15 AND 16.—On May 9 they were injected intracerebrally with filtrate of brain of rabbit 9.

June 6: Rabbit 15 died. The brain showed foci by well marked lesions. Rabbit 16 alive and well (Sept. 26).

MONKEY 12 (Ringtail Lemur Catta).—It was injected subdurally with mixture of filtrates of brains of rabbits 8 and 9.

May 9: Paresis of right arm noted.

May 13: Very ill. Paresis persists. Temperature elevated. Killed. Marked congestion of entire brain. Many punctate hemorrhages around site of injection. No injury to cerebrum. Punctate hemorrhages in midbrain and at base of brain. Lesion in cortex near motor area shows large hemorrhage surrounded by numerous smaller hemorrhages. Tissue is markedly edematous and contains many mononuclear leukocytes. Dilation of perivascular spaces with infiltration of mononuclear leukocytes. Foci of mononuclear leukocytic infiltration in cortex. Region of internal capsule on left side shows multiple hemorrhages with softening. Scattered foci of mononuclear leukocytes. Adjacent brain tissue is edematous. Marked dilation of perivascular lymph spaces with infiltration of mononuclear leukocytes. Meningitis of moderate intensity with mononuclear leukocytes. In cerebellum there is a small microscopic focus of mononuclear leukocytes.

RABBITS 29 AND 30.—On May 21, 1919, they were injected intracranially with filtrate of brain of monkey 12.

May 24: Rabbit 29 died after running course of lethargy for 3 days, during which time it was artificially fed. It was able to swallow if stimulated. Breathing shallow, but regular. Spasticity of both hind limbs noted on first day; flaccid throughout toward end. There were congestion; meningitis with mononuclear cell infiltration; focal infiltration in cortex of mononuclear leukocytes; perivascular and focal infiltration in basilar ganglions; minute microscopic hemorrhages in region of pyramidal fibers in medulla.

June 17: Rabbit 30. Paresis of both hind legs; opisthotonos; apathy.

June 24: Paresis more marked; increasing stupor and death. Very marked typical lesions throughout brain and cord.

EXPERIMENT 2

RABBITS 55 AND 56.—On June 25, they were injected intracranially with filtrate of nasopharyngeal mucous membrane of control fatal surgical case No. 1.

August 2: Rabbit 56 died. Brain negative. Intense bronchopneumonia.
September 26: Rabbit 55 alive and well.

RABBITS 59, 60 AND 61.—On June 15, they were injected intracranially with filtrate of nasopharyngeal mucous membrane of a fatal surgical case No. 2.

September 26: All rabbits alive and well to date.

RABBITS 11 AND 12.—On May 2 they were injected intracranially with filtrate of nasopharyngeal mucous membrane from rabbit 4, which succumbed to intracranial inoculation with filtrate of nasopharyngeal washings of patient.

May 3: Rabbit 11 died. Examination revealed marked congestion; meningitis with infiltration of mononuclear leukocytes; focus of infiltration in cortex with mononuclear leukocytes; area of hemorrhage and edema.

May 6: Rabbit 12 died. Examination revealed punctate hemorrhages over convexity of cerebrum and at base; intense general congestion; small hemor-

rhages in the cortex with a focus of mononuclear leukocytic infiltration; area of mononuclear leukocytes in one of basilar ganglions; and tense parivascular infiltration with mononuclear leukocytes.

RABBITS 17 AND 18.—On May 9, 1919, they were injected intracranially with filtrate of brain of rabbit 11.

May 28: Both alive and well.

RABBITS 19 AND 20.—On May 9, 1919, they were injected intracranially with filtrate of brain of rabbit 12.

May 10: Rabbit 19 died. There was slight congestion of brain; extensive bilateral bronchopneumonia and no abnormalities in brain microscopically.

August 14: Rabbit 20 died. Extensive bronchopneumonia. Mild meningitis.

RABBITS 57 AND 58.—On June 14 they were injected intracranially with filtrate of nasopharyngeal mucous membrane from a normal rabbit.

September 26: Both rabbits alive and well to date.

EXPERIMENT 3

RABBITS 27 AND 28.—On May 5, 1919, they were injected intracranially with filtrate of brain of monkey 2. (This brain had been kept two months in 50 per cent. glycerin solution.)

May 14: Rabbit 27 died. There was slight congestion; no meningitis, no perivascular infiltration, no hemorrhages and no focal infiltration.

May 17: Rabbit 28 died. Examination revealed congestion; moderate meningitis with foci of mononuclear leukocytes; tremendous congestion and dilatation of vessels of pia; very large focus of mononuclear cell infiltration about cortical vessel—vessel practically closed by the infiltration; numerous foci of mononuclear leukocytes scattered throughout cortex and at base of brain; marked congestion of cortical and subcortical vessels.

In every instance the material used for inoculation was cultured aerobically and anaerobically by the usual methods, and also by the Rosenow method, all with negative results. Studies with the Noguchi method of cultivation of filtrable viruses are in progress and the results will be reported later.

CONCLUSIONS

A filtrable virus was obtained from the nasopharyngeal mucous membrane of fatal cases of epidemic encephalitis. The virus is capable of producing in monkeys and rabbits lesions similar to those found in the human brain.

The virus has been carried through four generations in rabbits, transmitted to a monkey in the fifth generation, and then brought back to rabbits.

The virus can be recovered from the nasopharynx of animals inoculated intracranially.

A natural immunity was found in approximately 50% of our rabbits.

An acquired immunity was demonstrated in monkey 4.

A possible connection of this disease with influenza was hinted at in our previous report and studies are now being undertaken to establish such relationship.

NOTE.—Since this publication was submitted, further successful inoculations have been carried out in monkeys and rabbits. Additional control experiments were performed with human and rabbit nasopharyngeal mucous membranes. Typical brain lesions were produced in rabbits by means of filtrates of the nasopharyngeal mucus membrane from several of our positive experimental animals.

One of our strains which in the earlier inoculations manifested a tendency to produce hemorrhages, in subsequent inoculations yielded the more varied lesions found in human brains.

Cerebrospinal fluids from a fatal case of encephalitis caused the disease in rabbits. Transfers from brain to brain through filtrates have been successful in four generations up to the present time. Studies are being continued in this direction.

In the preliminary note appearing in the *Journal of the American Medical Association* for Oct. 4 we describe an organism isolated from the virus.

THE ROLE OF THE PFEIFFER BACILLUS IN THE RECENT EPIDEMIC OF INFLUENZA

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Since the recent visitation of influenza, which has been pandemic throughout the greater part of the civilized world, much has been written relative to its etiology and in particular to the part played by the influenza bacillus. While it is universally admitted that the pandemic disease of 1918-19 has not differed in any essential pathologic or clinical feature from that of the pandemic of 1890-92 when the cause was regarded as definitely established by Pfeiffer,¹ there is now considerable doubt, apparently, as to the etiologic relationship between clinical influenza and the Pfeiffer bacillus. Probably the main incentive for questioning the causal rôle of the Pfeiffer bacillus in the recent pandemic has been the severity and almost overwhelming clinical aspect of the disease prevalent in the infection. One was inclined to regard the picture as ascribable to some very virulent micro-organism or virus out of all accord in intensity of infection with the category of sporadic clinical entities promiscuously termed influenza, grip, and the like, usually noted in normal times. It must be recalled, however, that extraordinary circumstances existed in the massing of man power which formed the most desirable condition for intensifying the virulence of pathogenic microbes. Such an exaltation of the invasive power would explain the malignant character of *B. influenzae* when extending from military confines to adjacent municipalities. It must be remembered that the loss in virulence of greatly attenuated pathogenic species, long in stock as laboratory cultures, can be regenerated or exalted to a marked degree by animal passage. Certainly, in the conditions existing in military concentrations, the most desirable circumstances for repeated human passage were present.

In brief, there are three views extant today regarding the relationship of *B. influenzae* to the epidemic disease: (1) The acceptance of

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¹ Ztschr. f. Hyg. u. Infektionskrankh., 1893, 13, p. 357.

Pfeiffer's work and that of numerous confirmations by other reliable investigators;² (2) the view that the influenza bacillus plays only the rôle of a secondary invader, and that the disease is primarily caused by a filterable virus;³ and a third view, that the Pfeiffer bacillus, together with other pathogenic micro-organisms of the respiratory tract flora, produces conjointly the syndrome called influenza.⁴ Since the medical press has contained so many conflicting views concerning the etiology of epidemic influenza and the significance of various bacteria in the respiratory tract (the main dispute being over the part played by the Pfeiffer bacillus), we believe that our contribution to the study of the etiology will not be amiss at this time.

Our study is based on numerous cases of influenza in various hospitals in New Orleans during the primary and secondary outbreaks of the infection. Among the number are included 75 Porto Rican sailors ill with the disease, who were selected for study especially because they offered unusual opportunities in that they were all from the same source and clinically presented the same infection. Furthermore, the number was sufficient to form a basis for proper deductions from the results obtained. At the time of admission, approximately one-third of the number were apparently in the early stages of the disease, stating they had been stricken the day previous to their arrival in port, while others gave a history of being sick for a longer period. Several of these patients presented a well developed pneumonia. While under observation 17 of the 75 died and necropsies were made. Fifty-eight of the cases recovered after a period of from 2 to 6 weeks, and were discharged well.

The work consisted in (1) making cultures of the sputum, blood and tissues, especially from the lungs of those on whom necropsies were held; (2) gross and microscopic observations of the lesions found postmortem; (3) repeated blood examinations during the course of infection; and (4) serologic studies. In conjunction with the work approximately 5,000 persons were vaccinated with the protein of

² Weichselbaum, A.: *Wien. klin. Wchnschr.*, 1892, 32, p. 459. Huber, cited in Kolle und Wassermann: *Handbuch der Pathogenen micro-organismen*, 1903, 3, p. 359. Baumler, cited in Kolle und Wassermann: *Handbuch der Pathogenen micro-organismen*, 1903, 3, p. 359. Robertson, W. F.: *Brit. Med. Jour.*, 1918, 2, p. 680. Opie, Eugene L.; Freeman, A. W.; Blake, F. G., Small, J. C., and Rivers, T. M.: *The Jour. Am. Med. Assn.*, 1919, 72, p. 556. Spoener, L. H.; Scott, J. M., and Heath, E. H., Jr.: *Jour. Am. Med. Assn.*, 1919, 72, p. 155.

³ Nicolle, Charles, and Lebaillly, C.: *Compt. rend. Acad. d. sc.*, 1918, 167, p. 607. Dugarrie de la Riviere, R.: *Compt. rend. Acad. d. sc.*, 1918, 167, p. 606. MacCallum, W. G.: *Jour. Am. Med. Assn.*, 1919, 72, p. 720. Gibson, H. G.; Bowman, F. B., and Connor, J. I.: *Brit. Med. Jour.*, 1918, 2, p. 645.

⁴ Lord, F. J.; Scott, A. C., and Nye, R. N.: *Jour. Am. Med. Assn.*, 1919, 72, p. 188. Whittingham, H. E., and Sims, Carrie: *Lancet*, 1918, 2, p. 865.

killed influenza cultures and as the results have some bearing on the etiologic relationship of *B. influenzae*, certain aspects will be discussed.

BACTERIOLOGY

The sputum and blood were collected from all the cases and within two hours were plated (surface seeded) on complement free, human blood agar, 0.6% acid. Smear preparations from the specimens of sputum were also made as a tinctorial and morphologic index to the number of bacterial varieties in colonies on the plates. In this connection, however, it may be said that little dependence could be placed on bacterial morphology in the smears of the sputum as an aid in the identification of subsequent colony species because relatively small amounts are microscopically examined as compared to that planted, and furthermore morphologic features *in vivo* are not always repeated *in vitro*. The identity of the Pfeiffer bacillus was established by cultural characteristics and the behavior of the isolated culture toward known immune serum, employing for this purpose the agglutination and complement fixation tests.

The sputum from the 75 Porto Ricans yielded in every instance a bacillus corresponding in all essential characteristics to the micro-organism described by Pfeiffer. As a general rule, however, it was not the predominating colony on the plates, not even in instances of early infection. *B. influenzae* colonies were on the average more numerous on the plates from the cases of early infection than from those more advanced. In the latter we invariably encountered greater numbers of pneumococci and streptococci, which fact we attributed to the advent of secondary invasion in these cases.

The influenza colonies were slow in appearing on the plates, often not visible even with a hand lens under 48 hours incubation, while other bacterial species were well advanced or had by this time completed their colony growth. The slowness with which the influenza colony appears in its initial growth is to be anticipated in all work on its isolation from contaminated human material, and especially sputum, which usually contains a rich saprophytic flora. The mature influenza colony at best is often relatively minute as compared with those of other associated species, and in consequence may be overlooked unless careful attention is paid to this point. Not infrequently, when streaks were made in the seeding of plates, we noted merely a roughened tract or loss of surface gloss which on examination with a hand lens revealed numerous closely set, pin-point, transparent colonies of *B. influenzae*. We regard inattention to these points as probably accountable for the numerous reports of failure to isolate *B. influenzae* from the epidemic cases. Even where numbers of *B. influenzae* are present they may be inhibited in their development by the alterations in the medium brought about by the growth of other species. The antagonism in this respect is especially marked in the instance of the pneumococcus and streptococcus. On the other hand, we found the presence of the staphylococcus advantageous since it favored the growth of the influenza bacillus under artificial conditions.

We did not attempt the systematic isolation of the Pfeiffer bacillus from so-called normal throats or from the nose and mouth material of those suffering with other respiratory diseases; however, in those examined we occasionally found the Pfeiffer organism to which we attributed no significance as influenza was widespread throughout the community, and therefore its presence

was to be expected in many upper respiratory tracts, normal and otherwise.⁵ The incidence in normal throats and throats of recovered persons, however is interesting since it explains the survival through human carriers over the interval between epidemic outbreaks and the sporadic occurrence of the disease. The demonstrated occurrence of the meningococcus under similar circumstances⁶ forms an analogue in an accepted causative factor which readily permits of comparison. The finding of *B. influenzae*, therefore, in apparently normal throats months after the passing of an epidemic would not be evidence of any moment against its causal rôle in the disease known as influenza.

Blood cultures were negative for *B. influenzae* in all the cases examined; it was, however, recovered from the heart blood in three fatal pneumonias. In one it was present alone and in the two others associated with the hemolytic streptococci. It would seem that only occasionally, and then under conditions not entirely understood, the Pfeiffer bacillus gives rise to a septicemia.

The Pfeiffer bacillus was recovered from the pneumonic lungs at necropsy in 16 of 17 cases, but in none was it present in pure culture or even as the predominating species. We lay no stress on this point because the number of colonies developing *in vitro* is never an index to the number in the lesion as many at the time of plating may not have been viable, and others, highly parasitic, may not have been able to adjust themselves to the new artificial environment. The colonies on the plates were intermingled with those of the streptococcus, pneumococcus and staphylococcus, and occasionally with other undetermined bacteria species. The relative proportion of *B. influenzae* and those of the pneumo-streptococcal varieties were, as a rule, like that observed in the sputum of late cases of the disease. In certain of the cases wherein numerous small abscesses were scattered through the pneumonic lung, the predominating organism recovered was the staphylococcus aureus.

PATHOLOGIC ANATOMY

Since we are concerned especially with the relationship of *B. influenzae* to the respiratory lesion, only the tissue reaction of this system, and particularly that of the lungs, will be considered. The primary lesion of the respiratory tract is an intense acute nonsuppurative catarrhal inflammation of the mucosa and submucosa, involving the bronchial tree and often its finer terminals. These structures are tumefied and of a mottled dusky red color and the surface is moist and finely granular in appearance. The essential host reaction produced by *B. influenzae* corresponds in general to the gross picture occasioned locally in the tissues of animals following the injection of diphtheria toxin. This picture is later transformed to the ordinary gross lesion characteristic of secondary pyogenic invasion of the tissues.

Occasionally we encountered a rapidly fatal case in which the lung showed no gross evidence of consolidation but extensive areas in which the parenchyma was filled with a bloody fluid. There were usually present localized areas showing emphysema and dilatation of the bronchioles. Around the latter were grayish concentric patches which microscopically revealed a serofibrinous exudate. If the causal agent of influenza *per se* produces a distinct type of pneumonitis, this picture might be so regarded. On the other hand if this lesion is not to be attributed to *B. influenzae* it becomes necessary to regard it as the preneutrophilic or serum stage of exudation that represents the early

⁵ Pritchett, Ida, W., and Stillman, E. H.: Jour. Exper. Med., 1919, 29, p. 259.

⁶ Flexner, Simon: Mode of Infection, Means of Prevention and Specific Treatment of Epidemic Meningitis, 1917.

phenomena occasioned by secondary pyogenic invasion. The usual pneumonia seen at necropsy in our cases and regarded as produced by secondary infections, was definitely lobular in type, though in a few the extensiveness of the consolidation made it appear lobar on gross examination; however, on microscopic study its lobular character was established. As a rule, the pneumonitis with definite areas of consolidation presented nothing unusual either in the gross or microscopic appearance which would justify one in regarding the lesion as different from the lobular pneumonia caused by a variety of pyogenic micro-organisms. Our findings correspond in general to those of Wolbach,⁷ who describes two predominating types of pneumonia regarding them as different stages of the same process. However, he considers both types as caused by *B. influenzae*.

Multiple miliary abscesses occurred in many of the lungs seen by us at necropsy and not infrequently there was extensive hemorrhage in the lobes involved and myriads of petechiae on the pleura, which reminded us of the lung picture in bubonic plague. Our bacteriologic findings would indicate in these instances that secondary invaders are responsible for the ordinary pneumonic complication present and not the influenza bacillus per se.

LEUKOCYTIC REACTION

Our observations on clinical influenza revealed in the early stages of the disease little, if any, response on the part of the circulatory leukocytes; in fact, at this period there was a definite leukopenia. When pneumonic complications occurred there was invariably a definite rise of the normal count; this increase is to be attributed to secondary infection. As there is a constant leukopenia early in the disease, and as leukocytosis does not occur until the pneumonia has been superimposed, it would appear that the latter was occasioned by a secondary invader and not by the primary excitant of the infection. Leukocytosis may exist without pneumonic involvement during the course of the disease wherein secondary invasion of the upper respiratory tract has occurred. The total leukocytic count remained below 10,000 in the uncomplicated cases while in those presenting secondary lesions the count reached as high as 18,000. In the very early cases of influenza the counts were usually below 6,000. In the instances where there were complications with high leukocytic counts, the increase was due to the polymorphonuclear neutrophil.

SERUM REACTIONS

The blood from about 200 influenza patients representing all stages and periods of the disease, was tested for agglutination with the Pfeiffer bacillus. Varying dilutions of the patient's serum were employed, ranging from 1:20 to 1:100, and the final readings were made 24 hours after the mixtures were set up. The readings after from 18 to 24 hours have been found preferable because the agglutination reaction is slow, and readings made too early may therefore be misleading. The blood of patients, with few exceptions, gave very definite agglutination of *B. influenzae*, many reacting in dilution 1:80. Not infrequently the reaction was obtained as early as the third day of the disease. Normal blood used for control rarely reacted to the Pfeiffer bacillus and in the positive instances not in a serum dilution above 1:20.

The complement fixation test for the determination of specific lysin was also carried out on the series of cases. Specific lytic substance for *B. influenzae* antigen was not detected as a rule as early as the agglutinin; however,

⁷ Wolbach, S. B.: *Bull. Johns Hopkins Hosp.*, 1919, 30, p. 104.

in the later stages of the disease, particularly in those recovering, the reaction was fairly constant.

In our opinion the specific serum reaction obtained in influenza patients is deserving of more than passing interest. It has, perhaps, as great a significance from the standpoint of etiology for the Pfeiffer bacillus as any other postulate.

B. INFLUENZAE PROTEIN SPECIFICITY

The definite antigenic property of the Pfeiffer organism, as indicated by the serum behavior of patients and determined by animal experiments, led us to attempt the protection of the human against the infection with the specific protein of the freshly killed culture. Approximately 5,000 persons were vaccinated, each receiving three separate subcutaneous injections of 1,000,000,000 organisms at intervals of 3 days. The detailed report of the results obtained with vaccine will appear in a separate paper. Our intention here is to give only the points of interest which have a bearing in the establishment of a causal rôle for *B. influenzae*.

In the preparation of the specific protein several isolations of *B. influenzae* from recent cases of the disease and a culture from the Rockefeller Institute, which we have maintained as a stock culture for approximately five years, were employed. Wide variations in the immunizing power of the killed influenza culture depended to a large extent on the manner in which the protein was prepared. Heat as an agent for destroying the viability, altered in a greater or lesser degree the antigenic property. Likewise tricresol and other derivatives of the phenol group affected the immune-body production of killed *B. influenzae* protein. The maximum potency of the vaccine was obtained with chloroform as the bactericidal agent. This chemical apparently does not affect in the slightest degree the toxicity and because of its volatile property is quickly eliminated from the killed protein product.

The injection of the protein material gave a decided local and constitutional reaction in fully 90% of those vaccinated. In 30% the reaction was strikingly similar to that noted in influenza infection. A number of those inoculated were obliged to go to bed in from 6 to 8 hours after the administration of the first injection and to send for a physician who not infrequently pronounced the case influenza which subsequently proved to be only the severe constitutional reaction occasioned by the specific foreign protein. In general, it can be stated that the reaction ranged from a slight headache and mild pains over the body with a temperature of one-half to one degree F., lasting not more than 24 hours, to that of severe headache and neuralgic pains, ushered in by nausea, vomiting and chill and temperature of 101-102. It is noteworthy that in persons showing the more severe reactions the duration was shorter than in those in whom it was less severe.

In addition to the constitutional reaction there occurred a local transient inflammation at the site of inoculation. This ranged from a mild circumscribed erythema, 4-5 cm. in diameter to a markedly swollen and reddened skin involving the whole arm and greater part of the forearm. However, the intensity of the reaction was no index to the degree of constitutional effect from the vaccine. Often the constitutional reaction was associated with but little local inflammation at the inoculation site and conversely, the person developing a reddened and severely swollen arm often showed little fever and complained of no other indisposition.

The blood of the vaccinated without exception revealed the antigenic property of killed *B. influenzae* in the form of specific agglutinin in 36 hours after the first injection. This substance increased in the circulation to a significant degree after the second and third inoculations.

SUMMARY AND DISCUSSION

The work herein described had for its purpose the investigation of the specific pathogenicity of the organism known as *B. influenzae*. Based on a careful study of a representative series of influenza cases, we believe there is adequate proof to show that the Pfeiffer bacillus or a possible allied strain is the cause of the disease known as influenza which occurred in epidemic form in the early fall and winter of 1918. It would seem that sufficient postulates for the recognition of its etiology have been fulfilled.

In our opinion the influenza bacillus may be recovered from all cases of epidemic influenza. It was obtained from the sputum in every instance, and from the pneumonic lungs in 94 per cent. of the cases in which necropsies were held. Cultures from the heart blood yielded negative results in all except three of the fatal pneumonias. Here in addition to *B. influenzae* there was recovered the pneumococcus, streptococcus, staphylococcus and other undetermined species. In all cultures from the sputum and pneumonic lungs we encountered greater numbers of the pneumococcus and the streptococcus than we did *B. influenzae*. As the upper respiratory tract has normally a rich bacterial flora, it was not surprising to have some one or more of these organisms overshadow *B. influenzae* to such an extent that the isolation of the latter was accomplished with difficulty. It cannot be denied that the Pfeiffer bacillus is far more parasitic than any of the ordinary species found in the upper respiratory tract, which often accounts for the comparatively few colonies of *B. influenzae* that develop, or their failure to grow on special cultural media. Not infrequently attempts to isolate the Pfeiffer bacillus from the sputum of the late stage of the infection are unsuccessful because the organism is either there in too few numbers or is no longer viable, having been crowded out by the less parasitic and in consequence antagonistic bacteria of the normal flora. Again, the Pfeiffer bacillus in certain sputums may not be hardy enough to survive the change of environment produced by conditions of artificial cultivation, especially when transplanted with relatively large numbers of other less parasitic bacteria.

The finding of *B. influenzae* in the so-called normal nose and throat, and in various other lesions of the respiratory tract, which has been advanced by some⁸ as evidence against its specific causal relation to

⁸ Kinsella, R. A.: Jour. Am. Med. Assn., 1919, 72, p. 717. Lord, F. T.; Scott, A. C., and Nye, R. N.: Jour. Am. Med. Assn., 1919, 72, p. 188.

influenza, cannot from this standpoint be seriously considered. It has been definitely established that the bacillus may sojourn in the upper respiratory tract for months after recovery from the infection; and, furthermore, in the light of our knowledge of human carriers of other bacteria, such as *B. diphtheriae*, meningococcus, etc., it is entirely consistent with parasitism for persons not susceptible to harbor the parasite for a longer or shorter time.

In our opinion influenza begins primarily in the mucous membrane of some part of the respiratory tract and may spread rapidly from the original focus to the mucosa of the nasopharynx, larynx, trachea, bronchi and bronchioles. While the disease is essentially a toxemia, there occasionally occurs a bacteremia which would account for certain extrarespiratory lesions other than those not due purely to the specific toxin or secondary pyogenic invaders.

The microscopic changes indicate that the pneumonitis with definite consolidation is a superimposed lesion and not produced by the Pfeiffer bacillus per se. It is commonly the result of secondary pyogenic infection which is brought about by the injury to the tissues of the lower respiratory tract, occasioned by the influenza bacillus. While an acute inflammatory exudate is the common finding in the mucous membrane, we do not believe it represents the true lesion occasioned by *B. influenzae* but rather that produced by secondary invaders. The neutrophilic type of lesion in the respiratory tract is not surprising when we consider that normally in this situation there is a rich bacterial flora, members of which under a variety of conditions are capable of acting as secondary excitants. In consequence of the primary injury to the mucous membrane secondary infectious processes are common, and in particular those due to the pneumo-streptococcal group. These organisms are accepted as secondary invaders in other pathologic states of the air passageways; for example, in diphtheria and the acute exanthems, and therefore it is consistent to consider that they can play the same rôle in influenza.

The lesion in the respiratory tract of influenza in consequence of its site does not represent the true tissue reaction caused by any one pathogenic excitant, but the composite picture produced by a number of micro-organisms. Therefore, in describing the lesion one must not attempt to characterize it as pathognomonic of a particular entity. To form any idea of the specific host reaction to influenza infection one must study the changes in tissues that are removed from possible

source of confusion occasioned by coexisting micro-organisms. For example, pure influenza meningitis would afford a more desirable opportunity to observe the uncomplicated tissue reaction. Here it is plain that the essential change is lymphocytic and associated with proliferation.

Although the tests in animals with the living Pfeiffer bacillus have not been accepted as entirely satisfactory, certain results obtained by us in man with the injection of the chloroform killed influenzal cultures has definitely demonstrated its toxic qualities. During the epidemic we had occasion to vaccinate over 5,000 persons, and in fully 30 per cent. the constitutional reaction was strikingly similar to the early syndrome of the natural infection. We do not wish to infer that this reaction to influenzal protein is proof of the living organism's etiologic relationship; nevertheless, it is significant and therefore to be weighed in the consideration of the causal factor.

We regard the development of specific immune substances in the host during the course of the disease as of sufficient evidence to establish etiologic relationship. In practically all of our cases the agglutinin for the Pfeiffer bacillus was demonstrated and the lysin in some. The agglutinin was in evidence in the blood too early in the disease to be explained simply as the result of the activity of the Pfeiffer bacillus as a secondary invader. The specific agglutinin appeared often as early as the third day of the infection and rose steadily in amount as the disease progressed for those who made an uneventful recovery. In the rapidly fatal pneumonic cases the agglutinin was relatively low, and in several not detected. Controls usually gave no reaction for the Pfeiffer agglutinin in those who had not recently had influenza.

CONCLUSIONS

The micro-organism known as the bacillus of Pfeiffer may be isolated from the material of the respiratory tract lesion in all cases of epidemic influenza, and recovered only occasionally from persons not infected.

There is inadequate proof that the Pfeiffer bacillus is a member of the normal upper respiratory tract flora. That it may occur in normal individuals during epidemic times or persist for months in those who have recovered from the infection, is well recognized. These persons are the interim carriers of the bacillus and constitute an important means for transmission of the infectious agent.

During the course of the infection and for a variable time after recovery, the blood contains specific immune bodies for *B. influenzae* while those not infected are without these substances.

The subcutaneous inoculation of persons with influenza protein causes the production of specific immune bodies. The reaction occasioned in the inoculated person is definite evidence of its toxic property.

Secondary infections with one or more of the ordinary respiratory tract flora is common in epidemic influenza and is usually responsible for the occurrence of pneumonia.

The Pfeiffer bacillus is the primary cause of epidemic influenza for the reason that sufficient postulates in the recognition of its etiology can be fulfilled.

FURTHER STUDIES ON THE INFLUENZA EPIDEMIC AT CAMP GRANT

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The epidemic of influenza at Camp Grant reached its decline by Oct. 18, 1918, but for some time afterward the death rate in the hospital from respiratory diseases or their complications continued to be high. As similar epidemics had been, and at that time were, rampant in many camps and civil communities, it seemed important to continue the studies instituted during the epidemic.¹ Such an investigation would throw light on the bacteriology of any subsequent recrudescence of acute respiratory infections in a military camp after an influenza epidemic with bronchopneumonia. Due regard must be taken of the transient stay of the soldiers in this camp soon after the armistice, Nov. 11, 1918, and while patients were admitted to the hospital with the clinical diagnosis of influenza, only during the few weeks after the epidemic proper were the clinical course and the severity of symptoms the same as in patients admitted during the main epidemic.

Starting in December and continued into March of this year, 455 throat cultures of patients admitted for influenza were studied on plain blood agar according to the methods used earlier.

TABLE 1
RESULTS OF STUDY OF THROAT CULTURES OF INFLUENZA PATIENTS

Gram-positive, lancet-shaped diplococci, sometimes in chains, growing in green colonies on plain blood agar in.....	71.87%
Hemolytic streptococci	21.98%
Nonhemolytic streptococci	9.01%
<i>B. influenzae</i>	19.58%
<i>M. catarrhalis</i>	72.31%

Of the gram-positive, lancet-shaped diplococci, 169 pure cultures were isolated and studied more carefully; 19 were soluble in bile and fermented inulin, 43 were soluble in bile only, 45 fermented inulin only, and 32 gave no reaction with either of these tests.

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¹ Hirsch and McKinney: Epidemic of *Pneumococcus Bronchopneumonia*, *Jour. Infect. Dis.*, 1919, 24, p. 594.

The anatomic changes observed after death, when the epidemic had subsided, were chiefly such as complicate or follow pneumonia. Empyema was the commonest, usually in patients in whom the purulent exudate in the chest had been drained. Acute suppurative pneumonia with multiple peribronchial abscesses without definite limiting membranes was fairly common. The amount of destruction in such lungs varied widely, in some practically all the respiratory tissue of a lobe or a lung having been destroyed. Hemolytic streptococci were recovered from the exudate of such lungs with great regularity. It is possible that all of these acute suppurative pneumonias were essentially such from the beginning, but there is reason for believing that some of them were superimposed on a primary pneumonia without suppuration, because the examination of sections from earlier stages of such lungs showed necrosis in the alveolar walls or ductuli alveolares, this necrosis being but a small part of a much larger consolidation with changes like those in pneumococcus infection. In addition acute suppurative leptomeningitis and acute thrombo-ulcerative endocarditis were observed a few times.

Much different were the results in deaths from acute respiratory infections that in no way could be considered a complication of the epidemic disease. This group contains the largest number. Here the lungs were mottled by many, comparatively small, dark red areas, the increased firmness being due largely to the escape of blood into the substance. Large amounts of bloody fluid escaped from the cut surfaces, and the pleural cavities contained from 100 to 500 cc of thin reddish-brown fluid. Hemolytic streptococci were recovered in pure culture from the lungs and heart blood. In other cases the lungs contained irregular, nodular consolidations several centimeters in dimension, representing an older stage, and culturally yielding a similar bacterial flora. These nodules do not resemble in close detail the consolidations observed during the height of the epidemic in that the surfaces of the latter at the same stage of development were more coarsely granular without the yellow focal areas of necrosis, and the fluid expressed from the tissues was more viscid and grayish-brown rather than the reddish-brown of hemolytic streptococcus infection. The early acute hemolytic streptococcus pneumonia is also quite distinctive from the pneumonia of the epidemic.

During the 5 months in which these observations were made, there were only three cases in which the typical lobar pneumonia was

approached. All of these were noted after March 15, 1919. Consolidations of entire or nearly entire lobes were observed, but these were confluent bronchopneumonia rather than lobar pneumonia. That lobar pneumonia should be submerged during the epidemic and for months after, is of great interest. There seems to have been some fundamental change in the reaction of the host toward the infecting agent, some difference in the activity of the invading organism, or change in the interaction of both. Having found the pneumococci recovered during the epidemic highly virulent in resistant animals, might not absence of typical lobar pneumonia be explained on the basis of heightened virulence?

Cultures of tissues and fluids taken postmortem afford valuable information in acute infectious diseases of the variety of pathogenic bacteria present, and correlate the lesions with the organism concerned. The methods used during the epidemic were continued with slight modification in this study. As a rule, the cultures were obtained within 6 hours after death, and usually were controlled by direct smears made at the same time, stained later by Gram's method and dilute aqueous fuchsin.

During the period when complications of pneumonia were observed postmortem, and again later in a number of acute respiratory infections, hemolytic streptococci were recovered in mixed and pure cultures. With the ascendance of hemolytic streptococci in cultures, pneumococci became less frequent, although there were a few examinations during the first 3 months in which the lung changes and results of cultures corresponded well with those of the epidemic. In all the cultures from various places, influenza bacilli were found only occasionally with other organisms, never in pure culture. Pure cultures of staphylococcus albus were recovered from diseased lung tissues, body fluids, and the spleen in two examinations.

The lung culture results are briefly as follows for 49 of the 89 cases studied in which death probably had resulted from an acute respiratory disease after the epidemic or from lung complications of the epidemic disease:

TABLE 2
LUNG CULTURE RESULTS

Hemolytic streptococci in.....	30
Pneumococci	20
Staphylococcus albus	11
B. influenzae	8
Nonhemolytic streptococci	2

This tabulation may be misleading in that each of the various organisms listed as being present was not regarded in every instance as being the predominant organism. Of the remaining 40 examinations, 31 presented no immediate reason for culture of the lung, 3 others were not tabulated because of an active tuberculosis, while in the remaining 7 an acute suppurative leptomeningitis was demonstrated, the spinal fluid of 4 containing pneumococci, and of 3 hemolytic streptococci.

TABLE 3
RESULTS OF THE HEART BLOOD CULTURES OF THE EIGHTY-NINE NECROPSIES

Hemolytic streptococcus, pure.....	26
Pneumococcus, pure	18
Staphylococcus albus, pure.....	2
Negative	16
Not cultured	27

These results indicate that with the decline of the epidemic and subsequently, hemolytic streptococci became important as invaders of tissues already diseased, and frequently provoked disease in tissues not the site of a preceding change.

The postmortem bacteriologic studies demonstrated the presence of hemolytic streptococci, pneumococci and staphylococci in diseased fluids and tissues. Many strains were inoculated intraperitoneally into white mice in measured fractional quantities of 24-hour blood-agar slant cultures according to methods given in the previous report. With the technic used, the results from testing 51 strains of hemolytic streptococci, 48 strains of pneumococci, and 4 strains of staphylococcus albus indicate that the white mouse is more susceptible to the pneumococcus than to either of the other two organisms. Very few strains of hemolytic streptococci and none of the staphylococci killed white mice in quantities smaller than 0.166 c c of a 24-hour blood-agar slant culture, while many strains of pneumococci killed mice in quantities as small as 0.00154 of a culture.

As the virulence of certain of the epidemic strains of pneumococci had been tested in rabbits and guinea-pigs, similar tests were made with strains recovered postmortem after the epidemic from diseased lungs that approached more nearly lobar pneumonia.

Lung Culture.—Pneumococcus type 2 bile soluble, fermented inulin, capsulated, injected intraperitoneally into three guinea-pigs in respectively $\frac{1}{4}$, $\frac{1}{2}$ and 1-24 hour blood agar slant culture amounts. Two pigs died ($\frac{1}{2}$ and 1 culture); four rabbits were given intravenously $\frac{1}{4}$, $\frac{1}{2}$, 1 and 2-24 cultures; all lived but the one given 2 slant cultures.

Heart blood culture from same necropsy with identical characteristics were injected as above into three guinea-pigs, killing them all, and into three rabbits in $\frac{1}{2}$, 1, and 1-24 hour culture, killing one rabbit (1-24 hour culture); organism recovered pure from dead animals.

Similar experiments were made with four strains of pneumococci recovered postmortem April 8, 1919, each one, type 4, bile soluble, capsulated, fermented inulin and killed mice in dilutions as high as 0.0000154-24-hour blood-agar slant culture.

Three guinea-pigs and three rabbits were given as in the previous experiments $\frac{1}{4}$, $\frac{1}{2}$ and 1-24 hour blood-agar slant culture of each strain. All lived except two pigs receiving $\frac{1}{4}$ and 1-24-hour slant culture of different strains; organisms recovered pure from dead animals.

Rabbits and guinea-pigs receiving 0.1-24-hour blood agar slant culture of the epidemic strains died within a very short time, but a full 24-hour blood-agar slant culture of strains recovered from lungs post-mortem with gray consolidations of lobar pneumonia since the epidemic failed constantly to kill such animals.

During, and especially with and after, the decline of the epidemic, hemolytic streptococci were recovered from diseased tissues and blood fluids. Of strains recovered from various places, 79 were classified according to their ability to ferment lactose, mannite, and salicin as suggested by Holman.² All but 8 of these strains fermented lactose and salicin, but did not ferment mannite. None of the strains fermented inulin or were soluble in bile.

SUMMARY

With the decline of the influenza epidemic at Camp Grant, the changes noted at postmortem examinations were chiefly such as occur with a healing or complicated pneumonia.

The postepidemic pneumonias differed from the epidemic in that changes commonly recognized as due to hemolytic streptococcus were frequent.

Occasionally the epidemic type of pneumonia was noted within three months after the epidemic, but more recently the classical gray lobar variety was observed.

The postmortem bacteriologic studies demonstrated a high incidence of hemolytic streptococcus infections, less frequently pneumococcus.

The virulence of pneumococci recovered from diseased lungs since the epidemic is less in rabbits and guinea-pigs than the epidemic strains.

Hemolytic streptococci recovered during and since the epidemic, according to Holman's classification belong chiefly to the pyogenes group.

² Jour. Med. Research, 1916, 34, p. 377.

THE ANTIGEN OF BACILLUS ANTHRACIS

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It having been shown in previous articles¹ that the antigens of many bacteria and cells consist of pure fats, it seemed advisable to inquire whether a spore-bearing organism such as *B. anthracis* would yield a similar antigen. For this purpose there was selected a laboratory strain of anthrax bacillus, and a strain sent me from the Hygienic Laboratory at Washington. These strains were pure, the one a meager, the other an abundant, spore producer, and were easily raised in virulence for guinea-pigs and rabbits. While the method of isolation of fatty antigens has been given before, it will be briefly summarized.

Mass cultures of the anthrax strains were grown in Roux flasks on fat-free beef peptone agar, + 0.5, at 37 C. for from 24 to 36 hours. Very heavy cultures were obtained. The growth was washed off with sufficient distilled water and transferred to large florentine flasks. To the contents of the flasks were then added KOH in slight excess and sufficient alcohol to make about 40% water-alcohol mixture. The flasks were then gently heated on water baths under reflux condensers until hydrolysis was complete as shown by clear homogeneous yellow-brown contents. The presence of unsaturated fats in anthrax being shown by the gram positive stain of the organism, further steps were carried out with the view of preventing oxidation of these fats so far as possible. The alcohol was then distilled off in vacuo and the remaining contents of the flasks concentrated to a small volume with heat under 70 C. under diminished pressure, and the fats liberated as fatty acids by acidification with 20% sulphuric acid.

After cooling, the fatty acids were shaken out with ether. The unsaponified matter was collected and again saponified with KOH-alcohol, concentrated, acidified, cooled and extracted with ether, and the process repeated until a sample of ether showed no residue on evaporation. The portions of ether were then added together, concentrated to dryness in vacuo, resaponified and then reconverted into fatty acids that were dissolved in a small quantity of ether. To this ethereal solution was then added several volumes of absolute alcohol and the whole filtered, decolorized as far as possible with animal charcoal, filtered clear, concentrated to dryness in vacuo and weighed. On the average, 200 Roux flasks of culture should yield a minimum of one gram of fatty acids, and it is desirable to obtain not less than 5 grams for purposes of observation.

Having learned from previous experience that gram-staining organisms possess unsaturated fats, and that bacteria which most readily undergo autolysis

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¹ Jour. Infect. Dis., 1918, 22, p. 133.

contain considerable quantities of soluble volatile fats, while the most resistant species appear to have their fats composed largely of the more stable, saturated, nonvolatile types, it was suspected that the anthrax bacillus would be found to contain for the most part fats of the first two types, unsaturated and volatile, because of its gram-positive character and for the reason that the organism readily autolyzes at the period of sporulation. Accordingly, the fatty acids were subjected to distillation in steam until all volatile acids had passed over into the distillate. These acids were separated from the acid water by extraction with ether in the presence of solid NaCl. The ethereal solution of soluble volatile acids constituted portion A.

The remaining nonsoluble fatty acids, showing a melting point of 20-21 C., were then divided by the lead salt-ether method into portions B, a very small one consisting of saturated fatty acids, and C, containing the unsaturated fatty acids.

The acids of portion A were then converted into their methyl esters and distilled in vacuo, by which process two fractions were obtained, one distilling at 96 C. at 15 mm., the other at about 114 C. These fractions were then saponified with KOH and the respective fatty acids liberated, dissolved in ether, washed, dried, filtered and weighed. The smaller fraction was fluid at 20 C. but solidified at about 14-16 C. The neutralization value approximated 390 mg. KOH from which the calculated molecular weight would be about 150. This acid corresponded very closely with caprylic acid.

The larger portion, about 60%, or 75%, showed a melting point of 30 C., a neutralization value of 320 and a molecular weight of 170, corresponding fairly closely to capric acid. The melting point of the combined portions of A, it was stated, was 21 C. When one is dealing with a fairly narrow fraction of fatty acids, such for instance as compose portion A, it is helpful and quite permissible to draw inferences concerning the identity and proportions of the ingredients providing certain data are at hand. The mixture of volatile acids, one of which was known to be fluid at temperatures above 16 C., was regarded as consisting of about $\frac{1}{4}$ caprylic acid and $\frac{3}{4}$ capric acid before the fractional distillation was done, since a previous observation of melting points of combinations of volatile acids had recorded 20 C. as representing this particular combination. With combinations of solid, saturated acids above capric such inferences are hardly practicable since they form eutectic mixtures having often a melting point lower than either of the ingredients.

Portion B was too small to work with, but by difference it was found to make up about 5% of the total.

Portion C, a liquid fatty acid, gave a neutralization value of 200 and calculated M.W. of 280. The iodine value by the Hübl method was 88. This acid corresponded closely enough to oleic, and constituted the larger part of the total. The melting point of this acid in combination with the saturated fraction, 20 C., would indicate the saturated acid to be one lower than stearic, since the melting point of 95% oleic and 5% stearic lies above that point, and it was accordingly assumed that this small saturated fraction might consist of palmitic acid.

From this tentative analysis, then, it appeared that the fatty complex of the anthrax bacillus would be approximately represented by oleic acid 65%, palmitic acid 5%, capric acid 20% and caprylic acid 10%. In order to determine the exact constitution and proportions

of the fatty acids existing as such, or as fats, or esters in the anthrax bacilli, recourse was had, as with the other antigens studied, to the delicate test of complement fixation with the serums of animals immunized with the germ bodies.

Rabbits were selected for immunization, and the fat complexes or antigens for testing were combined artificially, in varying proportions approximating that given by the analysis, from fatty acids of highest purity obtained from sources other than the germ bodies, some having been made synthetically and all being free from protein. Before being combined, the acids were converted into their sodium salts and the latter were dissolved in desired proportions in absolute alcohol in such strength that 1 cc of solution contained 2 mg. of combined salts. To each 1 cc of solution there was then added 0.8 cc of 1% alcoholic solution of purified cholesterol. These quantities and dilutions were employed because previous work had shown them to be proper for a considerable number of fatty antigens.

The complement fixation tests were carried out in a manner previously described, using as control antigen a heavy suspension of a 24-hour agar culture of anthrax bacilli in salt solution, and employing the rabbit antisheep-cell hemolytic system. Eleven antigens in all were tried, of which 6 were quickly eliminated, leaving 5 for more careful consideration, the compositions of which follow:

7. Caprylic acid 10%, capric acid 20%, oleic acid 50%.
8. Caprylic 20%, capric 20%, oleic 60%.
9. Caprylic 10%, capric 20%, oleic 55%, myristic 5%.
10. Caprylic 10%, capric 20%, oleic 65%, myristic 5%.
11. Caprylic 10%, capric 25%, oleic 60%, palmitic 5%.

The reactions of these antigens with the immune and control serums are shown in table 1.

TABLE 1
REACTION OF ANTIGENS WITH IMMUNE AND CONTROL SERUMS

Serum	Antigen 7	Antigen 8	Antigen 9	Antigen 10	Antigen 11	Anthrax Susten- sion	Typhoid Baillus Susten- sion
Rabbit, immune 1.....	++	++	+	++	++	++	—
Rabbit, immune 2.....	+	+	++	++	++	++	—
Rabbit, immune 3.....	+	+	++	++	++	++	—
Rabbit, immune 4.....	++	++	++	+	++	++	—
Rabbit, normal.....	—	—	—	+	+	+	—
Rabbit, normal.....	—	—	+	+	+	—	—
Rabbit, normal.....	—	—	—	—	—	—	—
Rabbit, normal.....	—	—	—	±	—	—	—
Human, normal.....	—	—	—	—	—	—	—
Human, normal.....	+	±	+	+	±	±	++
Human, syphilitic.....	—	—	—	—	—	—	—
Human, gonorrheal.....	—	—	—	—	±	—	—
Antigen control.....	—	—	—	—	±	—	—

Antigen 11 gave reactions most closely approximating the germ suspension antigen. This antigen was then tested further for specificity by the agglutination-precipitation test described in earlier papers. Exactly the same proportions were used in the test except for the addition to the antigen of a trace of egg lecithin which was added solely for its particular colloidal properties.

Of this stabilized antigen 0.07 cc was added to 0.5 cc of salt solution in each determinant and control tube and the mixture shaken. Each tube then received 0.28 cc of the inactivated serums to be examined. After shaking again the tubes were kept in the icebox for from 8-10 hours. The precipitations are recorded in table 2.

TABLE 2
RESULT OF AGGLUTINATION-PRECIPITATION TEST

Serums	Fat—Antigens		
	Anthrax	Cholera	Typhoid
Rabbit, immune 1.....	++	—	—
Rabbit, immune 2.....	++	—	—
Rabbit, immune 3.....	++	—	—
Rabbit, immune 4.....	++	—	—
Rabbit, normal.....	—	—	—
Rabbit, normal.....	—	—	—
Human, normal.....	—	—	—
Human, normal.....	—	—	—
Antigen control.....	—	—	—

++ means complete precipitation, supernatant fluid clear.

— means no precipitation, homogeneous emulsion unchanged.

Artificial antigen 11 was then adopted for further work in the belief that the particular fat complex characteristic of the anthrax bacillus had been closely approximated, and the tentative proportions of the ingredients furnished by the analysis were corrected.

During the progress of observations on the antigen there developed what appeared to be, by a process of exclusion, a new means of testing its specificity, depending on the persistence of anthrax germs in the form of spores, whereby animals were made to develop spontaneous anthrax (autoinoculation) following specific lowering of resistance by injection of large doses of the artificial fat antigen. For this purpose guinea-pigs lent themselves most readily. Before taking up the experiments connected with this phase of the work it is necessary to discuss the sterility of the antigen.

During the work on the fatty antigens it has been the custom to preserve the sodium salts of the various fatty acids, freshly prepared, used in the preparation of the antigens, including anthrax, in stock solutions in absolute alcohol, from which the various complexes are prepared as needed. Repeated cultures of these solutions, and of the salts prior to their solution, have shown them to be entirely free from bacteria or spores of any kind. After combining the ingredients of the anthrax antigen, this alcoholic solution was again thoroughly tested for sterility. For fluid cultures 10 and 40 cc quantities of beef-peptone broth + 0.5, containing 0.2% glucose, were employed into which were placed graduated amounts of the alcoholic antigen from 0.01 cc (0.00001 gr.) to 5 cc. Cultures of the solution were also made on nutrient and blood agar under aerobic and anaerobic conditions. Portions of the alcoholic antigen solution were then concentrated to dryness and corresponding weights of the dry salts were subjected to culture in the same manner. All cultures were invariably sterile.

Later these tests for sterility were carefully repeated. It was found that the dose of the antigen, in 10 cc quantities of broth, which inhibited the growth of anthrax bacilli from spores, was 0.6 cc or 0.0006 mg. Cultures were then made of varying amounts of the antigen, in alcoholic solution and in powder, below the inhibiting dose, in 1 and 2 liter amounts of nutrient broth and these flasks were allowed to remain at from 35-37 C. for a month. As with the earlier tests, all cultures remained negative. After the long rest in the incubator, traces of any sediments in the flasks were then carefully pipetted off on nutrient agar. No growths occurred. Finally the antigen was filtered through a series of Berkefeld filters which were shown to be impervious to anthrax spores, and cultures of these filtrates were likewise sterile.

It is difficult to see how any germ or spore of anthrax, or of any other organism for that matter, could find its way into the antigenic materials. The absolute alcohol itself was above suspicion and the method of preparation of the antigenic powders and solutions precluded the possibility of anthrax or other contamination. As corroborative evidence of this fact, the following test was convincing: To the fatty acids entering into the antigen there were added 2 agar slants of anthrax culture 10 days old, consisting almost entirely of viable spores, and these mixtures were then subjected to the usual technic of conversion of fatty acids into the sodium salts. Of these solid salts, 50 mg. portions were inoculated into 1.5 liter quantities of nutrient broth. Of the alcoholic antigen solutions of these salts, varying amounts in duplicate were sown into 10 cc quantities of the broth. These cultures remained negative after one month's time and transplants on agar of any recoverable sediments as well.

The fatty acids themselves were of the highest purity obtainable, and were made some from vegetable sources, others synthetically. There remain to be considered the salt solution used in the dilution of the antigen for purposes of injection, the glassware, the syringes and needles, and the antigen solution ready for injection. The salt solution was prepared freshly in small amounts and autoclaved in flasks at 115 C. for 30 minutes. The pipets and other glassware were invariably sterilized in dry heat at from 200-220 C. The all-glass syringes and the needles when not in use were kept in a saturated solution of borax, and were thoroughly sterilized before using by long boiling. Cultures of the salt solution passed through the syringes, and the needles were sterile. The diluted antigen in salt solution ready for injecting was subjected, in a routine manner, to culture in broth and on agar for a long period, but no growth ever developed. In all the many hundreds of injections of numerous fatty antigens other than anthrax, similarly prepared, in animals and in man, no case of anthrax or other infection ever developed, and no case of spontaneous anthrax ever appeared in this laboratory, or in any other that the writer has learned of, until the use of the anthrax antigen was begun. It is certain then that every source of anthrax spores outside of the animals and their food has been excluded.

Exper. 1.—Four guinea-pigs, averaging 400 gm. in weight, received daily for 3 days subcutaneous injections of 0.5 mg. of artificial fatty anthrax antigen in 2 cc of salt solution. The pigs were kept in a metal-bottom, wire cage and fed on baled hay, carrots and stale bread or crackers. Two days following the last injection all the animals developed extensive subcutaneous swellings on the abdomen, became sick and died, 2 within 72 hours of the last injection, another on the 4th day and the fourth, one week after the first injection.

The necropsies all showed the same picture: Extensive subcutaneous, slightly bloody, gelatinous edema, in some areas 2 cm. thick; intestines slightly injected, the liver, and particularly the spleen, greatly enlarged, dark and friable; slight hemorrhagic exudate in the pleural cavity; lungs hemorrhagic in areas, with blood showing at the nostrils in some animals. The edema fluid contained in hanging drop fairly numerous long, large, nonmotile bacilli, sometimes single, often in short chains of 2 or more, the organisms having a definite capsule. Stained preparations from spleen fluid showed enormous masses of large gram-positive bacilli with slightly rounded free ends, but with square cut ends where joined. Cultures from the heart blood on nutrient agar under aerobic conditions at 35 C. yielded in from 12-24 hours heavy, flat, dull white ground-glass like growths which in microscopic preparations consisted of gram-positive spore-bearing bacilli. In nutrient broth the growth was less extensive, flocculent or in ropes, leaving the broth itself clear. The flocculi consisted of entwined chains of bacilli. In short, anthrax was obtained in pure culture from all animals, and the several cultures reinjected into fresh guinea-pigs and rabbits produced typical anthrax infections and death.

To determine if possible the source of the infection a similar series of 4 guinea-pigs was injected with fat antigen, and were housed in a wooden cage in an animal room in another part of the building, but were fed as before. This second series died in about the same interval of time and showed the same lesions and cultures.

A third series of 4 guinea-pigs was then selected from a large pen containing from 75-100 other pigs in still another room in the building. This series was injected as before, marked, and turned loose in the pen. All injected animals died of anthrax and yielded pure cultures, but none of the other guinea-pigs became infected.

A fourth series of 4 pigs was then injected with antigen as before and placed in an iron cage which, with its drinking vessel, had been sterilized in an oven at 200 C. for 2 hours. They were given the usual hay and other food. All the members of this series died of anthrax. These tests appeared to narrow the infection down to the food and the animals themselves.

Still another series of pigs was injected with antigen as before, confined in freshly sterilized glass jars and fed on crackers only. In all cases the water was the same as that supplied to the other animals in use in the same rooms. This series died of anthrax in 5 days, showing that probably the source of infection was within the bodies of the animals at the moment of the experiment.

Concurrently with these series of animals, similar groups of guinea-pigs were injected with diminishing doses of the fatty antigen, both subcutaneously and intraperitoneally. The results on the latter groups showed that from subcutaneous doses of $\frac{1}{4}$ of the original quantity, and from intraperitoneal doses of $\frac{1}{10}$ the amount many of the animals escaped infection, and with still lower doses the infections ceased altogether and signs of resistance to infection began to appear. It was also observed that spaced injections of the antigen intraperitoneally into guinea-pigs produced anthrax, the lowest doses being 0.01 mg. (corresponding roughly to that amount of antigenic substance contained in 6 loops of fresh anthrax culture). These facts appeared to indicate that specific sensitization varied directly with the antigenic dose.

For purposes of control the following series of guinea-pigs were used: 9 guinea-pigs, averaging 400 gm. in weight, that had received 3 subcutaneous injections of artificial gonococcus fatty antigen equal in frequency, weight

and bulk to the maximum anthrax doses. Four were placed in an infected wooden cage and 4 in a wire cage in separate rooms. No animal became infected and all were well in 14 days.

Eight guinea-pigs, averaging 300 gm. in weight, received similar subcutaneous injections of artificial cholera fat antigen and were kept in infected cages for 14 days. These animals remained well.

Five guinea-pigs, 450 gm. in weight, were injected daily for 3 days with equal weight and bulk of syphilis fatty antigen and kept in infected cages. No anthrax infections resulted.

Six guinea-pigs, 400 gm. in weight, that had received intradermal inoculations of rat serum and rat anaphylatoxin and had developed Arthus' phenomenon with local necroses, were placed in cages in which guinea-pigs had died of anthrax where they remained from 2-3 weeks without signs of infection.

Six guinea-pigs that had received even larger intraperitoneal injections of the fats of tubercle bacilli and that were placed in similar surroundings did not develop anthrax.

A group of 10 pigs of from 200-225 gm. in average weight whose resistance had been impaired by intraperitoneal injections of rat serum, 4 of 5 cc, 4 of 2.5 cc and 2 of 1.5 cc were placed in pairs in ordinary glass jars and fed on the ordinary diet of hay, etc. None of this group acquired anthrax.

In a general lowering of resistance, such as accompanies Arthus' phenomenon or the intraperitoneal injection of heterologous serums into guinea-pigs, death is commonly due to an infection by a sort of septicemic organism, called pseudopneumococcus by Nicolle, to which the animals are normally susceptible. This was seen in a few instances in the present study where the doses of the anthrax and other antigens had been small and where no other infections had developed. Where, however, the animals are markedly sensitized to anthrax there is usually no time for the ordinary infection to develop. In 2 pigs injected with small doses of anthrax antigen, simultaneous anthrax and pasteurella were observed.

If the guinea-pigs in this environment are normally carriers of anthrax spores, as seems reasonable to suppose, one would expect in the lowering of specific resistance by fat antigens other than anthrax to meet occasional instances where the general lowering of resistance would give rise to spontaneous anthrax, particularly since many of the antigens contain often one or more of the same ingredients in varying proportions. Accordingly those control groups injected with gonococcus, syphilis and other antigens have been scrutinized with considerable care in the course of this work, although in none of the experiments previous to and synchronous with the use of the anthrax antigen had anthrax developed. In one instance where a series of guinea-pigs had received enormous doses of gonococcus antigen an animal died of anthrax on the 2nd day following the last injection, and one animal in a series injected with corresponding doses of syphilis antigen (sodium oleate). Undoubtedly other such cases would appear in the long run. On the other hand, it was thought too that there should be found, here and there, certain pigs free from spores at the time of injection of heavy doses of anthrax antigen that would escape the infection, and still others, spore free, in which the infection would be delayed, the ingestion of spores occurring after the injection. The suddenness of the onset and the rapid course of the disease in nearly all the animals, in some of which the infection was already under way at the time of the third injection, left no doubt regarding the presence of

spores, but during repetitions of the observations on certain groups, several pigs were found in which the infection was delayed a week or more following the injections.

The supposedly different strains of anthrax bacilli picked up in this manner showed few or no morphologic or cultural variations, the chief differences being shown in broth cultures in which some strains grew in pronounced ropes, others in flocculi. In marked contrast to our old laboratory strain, all are good spore producers. None showed a virulence for guinea-pigs or rabbits greater than a single passage through an animal would account for. Many animals withstood subcutaneous doses of one or two loops of some of the strains.

Rabbits also acquire anthrax from injections of the antigen, but larger doses are required. As a rule, they are not so susceptible, and many rabbits injected with large doses escape infection. They succumb more readily to intraperitoneal doses of moderate amount spaced in 5 day intervals. Out of a series of 8 rabbits that had received 0.2 mg. of anthrax antigen in this manner for three doses, 4 died of anthrax, and 4 were shown later to be resistant to the injection of virulent culture.

Mice have not shown anthrax infection following intraperitoneal and subcutaneous injections of the anthrax antigen in doses equal to the minimum sensitizing doses for guinea-pigs.

For further purpose of control it was thought advisable to determine whether a protein derived from anthrax organisms would sensitize animals in a manner similar to the fatty antigen. For this purpose a protein was prepared and used in the following way: It was obtained from fresh 24-hour mass cultures of *B. anthracis* after the method described in an earlier paper.¹ After thorough extraction of the culture material with alcohol and with ether, the dry germ residue, called bacillary substance by Vaughan and Wheeler, was extracted with distilled water containing a trace of chloroform at 37 C. for 8 days, the undissolved mass removed by centrifugation at 8,000 revolutions per minute and the aqueous solution concentrated to dryness. At the same time, similar proteins were obtained from *B. typhosus*, and from *V. cholerae*. These proteins, soluble and nonheat-coagulable, and containing traces of amino acids, were fatal to rabbits in intravenous, and to guinea-pigs, of 300 gm., in intraperitoneal doses of 0.1 mg.

Eight guinea-pigs, averaging 300 gm. in weight, received 3 intraperitoneal inoculations at intervals of one day of 0.05 mg. of anthrax protein, the maximum dose consistent with safety. Following each injection the pigs were ill, but they apparently recovered within 2 days after the last injection. None of the guinea-pigs developed anthrax.

At the same time 6 rabbits were injected intravenously with the same doses and at the same intervals 2 each with typhoid protein, anthrax protein and cholera protein. Seven days after the third and last injection the 6 rabbits, together with 2 normal control rabbits, were bled from the heart, the serums separated and tested for precipitation with the various protein antigens. The serums of the normal control rabbits gave no precipitation with any antigen. The serums of the two typhoid rabbits precipitated with the typhoid, the anthrax and the cholera antigens; and the anthrax and cholera serums precipitated with their appropriate antigens and also with the 2 crossed antigens. The differences in the degrees of interprecipitation were not greater than would be accounted for by individual variations in animals. From this observation it was inferred that the 3 germ proteins prepared in this manner were probably similar in nature.

Fourteen days after the last injection—4 days after the bleeding—the 2 rabbits that had received anthrax protein were inoculated, simultaneously with 2 normal control rabbits, with the minimal fatal dose of fresh anthrax culture. All 4 rabbits died from anthrax infection within 4 days.

Exper. 2.—Having shown that probable foci of the anthrax spores exist within the bodies of the guinea-pigs and rabbits, it was thought that examination of the hay used as food would disclose the primary source.

From beneath a lifted corner of a partly used bale of hay a portion of dust was gathered from the cement floor and sifted through a flamed fine wire mesh. About 3 gm. of dust was placed in a sterile flask and shaken with 60 cc of sterile salt solution. The contents were then filtered through sterile gauze into sterile cups and centrifuged at 8,000 r. p. m. After pipetting off the clear greenish supernatant fluid, there were added to the sediment in each of two cups 2 cc of sterile salt solution. The sediment was then shaken and allowed to stand for 15 minutes to permit coarse particles to settle. From each tube one loop of fluid was passed into several tubes of melted agar and poured into plates. The balance of the fluid was injected into a series of white mice. These animals all died in from 5-8 hours from a general infection caused by a short, plump, bipolar staining, pestis-like organism. Anthrax bacilli were not found in slides or in cultures of the blood. The plates, kept at 37 C. for 24 hours yielded, among a variety of organisms, on an average one anthrax colony, though others may have been overlooked. Subcultures in broth and on agar yielded characteristic growths. One agar slant culture injected subcutaneously into 2 guinea-pigs of 400 gm., each receiving $\frac{1}{2}$ slant, was sufficient to kill the animals on the 4th day. The necropsies showed typical anthrax lesions, and cultures of the heart blood in broth and on agar developed pure *B. anthracis*.

The second part of this experiment consisted in the examination of the contents of the stomach of a normal guinea-pig. An animal weighing 350 gm. 3 hours after feeding on baled hay, was killed by anesthesia, the stomach opened aseptically and the contents turned out into a sterile flask with a wide mouth containing 60 cc of sterile salt solution. After thorough mixing and neutralizing to litmus with sterile sodium carbonate solution, the coarser contents of the flask were allowed to settle for one hour. The supernatant cloudy fluid was pipetted into sterile cups and centrifugated at 8,000 revolutions per minute. From this point the technic was the same as in the first half of the experiment and anthrax organisms were isolated from colonies on the plates and passed through guinea-pigs.

Exper. 3.—The observations on the anthrax antigen which was passed through Berkefeld filters, shown to be impervious to anthrax spores, prior to inoculation into animals, were made the subject of a separate experiment.

Filtration of the antigen emulsified in salt solution and ready for inoculation into guinea-pigs was, of course, impossible because of its particulate nature, practically all the antigenic material remaining on the surface of the candles. It was necessary then to filter the alcoholic solution.

A number of small candles of fairly fine texture were washed with ether, alcohol and water and shown to be free from fats. They were next autoclaved at 120 C. in water for 20 minutes, and then dried and again sterilized in the oven at 200 C. Through 8 of these filters there was then passed in divided quantities of about 18 cc, 150 cc of nutrient broth, in which had been thoroughly mixed one large agar slant of 10-day culture of anthrax, containing great numbers of viable spores. The filtrates, received into separate sterile containers, were kept at from 35-37 C. for 14 days and remained sterile.

The filters were again cleaned and sterilized as above, and through each one 15 cc of the alcoholic antigen solution were then passed. From each portion there was withdrawn sufficient antigen for purposes of culture, and into 2 liter quantities of broth were inoculated 5 and 50 mg. These cultures remained sterile after 30 days at from 35-37 C. In preparing emulsions from the filtrates for injection it was observed that their character had been changed by the filtration. Nevertheless, from the filtrates of 6 filters, 12 guinea-pigs were injected subcutaneously daily for 3 days with a quantity supposed to contain 0.25 mg. of antigen, a dose equal to half that found to lead to infection in nearly all instances. None of these animals developed anthrax within 21 days.

Following the filtration of the antigen, all filters were washed with alcohol and the concentrates of the washings showed merely traces of antigen. However, on washing the filters with water in a reverse manner, the water came through milky and these aqueous portions yielded relatively large amounts of fats. It was apparent, therefore, that the antigens had been changed and had lost some of their fat content by adsorption on the filters. In order to approximate the extent and character of the changes, filters *g* and *h* of the same series of 8, after being cleaned and prepared, were tested by the passage of 0.100 gm. of antigen in alcoholic solution. Suction was maintained until the filters were perfectly dry. Through each 20 cc of absolute alcohol were then passed, and these portions added to the filtrates. The combined filtrates and alcohol washings were then quantitatively concentrated, dried and weighed. Through filter *g* there passed 0.0648 gm. Filter *h* allowed to pass 0.058 gm. Filters *g* and *h* were then washed with water in a reversed manner until the fluid came through quite clear. From the washings there were obtained of dry substance, after concentration, filter *g* 0.033 gm. and filter *h* 0.037 gm. It was therefore apparent that the filtrates used for the injection of animals were short probably at least 40% of their antigenic material. An examination of the retained substances also indicated that sodium oleate had been retained in amounts greater, proportionately, so that not only had the antigen been below strength, but its chemical composition had also been somewhat altered. The fact then that none of the 12 guinea-pigs acquired anthrax occasioned no surprise but rather was in accord with previous experience.

It was then determined to ascertain whether increasing the dosage of the filtered antigen so as to make the amount injected equal to 0.25 mg., as computed from the figures obtained above, would induce infection despite the slight alteration in the fat complex. The original filtrates of *g* and *h* were then injected in the required increased amounts and at the usual intervals into a series of 5 guinea-pigs, 2 with *g* filtrate, and 3 with *h*. Two days after the last injection, one of the animals injected with filtrate *g* died from anthrax, and cultures were obtained from the heart blood. Two days later one of the guinea-pigs injected with filtrate *h* died from anthrax infection, and yielded positive cultures from the heart blood. The other animals of the group remained well.

Exper. 4.—This experiment deals with increased resistance in rabbits against virulent anthrax organisms following immunization with artificial fatty antigen.

A recently isolated strain of *B. anthracis* (Hygienic Laboratory), a good spore producer, which at first was not particularly virulent for rabbits, was raised in virulence during the course of 6 months by repeated passages through these animals until an intraperitoneal dose of 0.00001 of an arbitrary 2 mm. loop was invariably fatal to full-grown rabbits in 4 days.

Eight rabbits, averaging 2,000 gm., were injected intraperitoneally 4 times at intervals of 5 days with 0.00025 gram of artificial anthrax fatty antigen suspended in 1 cc of salt solution, an amount of antigenic material contained roughly in 2 square inches of fresh culture. Five days after the last injection, 4 of the rabbits died of spontaneous anthrax, and 4 survived. The date on which the last part of the injections was given was regarded as the low point in the depression of resistance, and the surviving animals were left undisturbed for 4 weeks. At the conclusion of this time each of the 4 rabbits, together with an equal number of control rabbits, received intraperitoneally the invariably fatal dose of anthrax culture. The control animals died within 4 days, whereas the determinants remained well. These 4 surviving rabbits, after an interval of 8 days, were then injected intraperitoneally with 0.0001 of a loop of fresh virulent culture. All died from anthrax within 4 days. This showed that the rabbits to have been protected against a fatal dose of culture, but not against 100 times the fatal dose—a great excess of culture. The 4 rabbits that died from spontaneous anthrax were mentioned earlier in this paper under the experiments on specificity of antigen.

A similar group of 8 rabbits received intraperitoneally 3 injections of anthrax fatty antigen at intervals of 4 days, the first injection containing 0.0000024 grain and the following injections containing two and a half times that amount. Two of the rabbits died with snuffles 72 hours after the first injection and showed no signs of infection by anthrax. Five weeks after the last injection the 6 surviving rabbits, together with the same number of control rabbits, received the fatal dose of fresh culture. All control animals died but the determinants remained well. Eight days later these rabbits were injected with 100 times the fatal dose of anthrax culture to which they succumbed within the usual time.

A third group of 6 rabbits received 3 intraperitoneal injections of 0.00003 gm. of anthrax antigen at intervals of 14 days.

A fourth group of 6 rabbits received daily for 3 days intravenous injections of 0.00006 gm. of anthrax fatty antigen. Fifteen days after the last injections, the last two groups being timed together, the 12 rabbits, with an equal number of control normal rabbits, received intraperitoneal injections of fresh virulent anthrax culture as follows: Of group 3, two rabbits received 2 fatal doses, 2 received 4 fatal doses, and 2 received 5 fatal doses. Of group 4, two rabbits were injected with 5 fatal doses, 2 with 7 fatal doses and 2 with 10 fatal doses. All control rabbits died, and of the 2 groups all survived save one of group 4 that had received 7 fatal doses. This animal died of anthrax on the 3rd day.

As further evidence of the presence of immune bodies in the blood of these animals, the serums of the 2 rabbits that withstood 10 fatal doses of culture were tested therefor. Two normal rabbits served as controls and also 2 rabbits that had been immunized against the cholera vibrio by 4 daily intravenous injections of 0.0001 gm. of artificial cholera fatty antigen, and bled 10 days later.

The active serums of the cholera rabbits, and the same inactivated in the presence of fresh guinea-pig complement, gave strong Pfeiffer phenomena, macroscopic and microscopic, whereas the serums of the normal and the anthrax immune rabbits were negative. On the other hand, the serums of the anthrax animals alone gave positive agglutination-precipitation reactions with emulsions of anthrax antigen in salt solution.

DISCUSSION AND CONCLUSIONS

Two phases of the work require a few words of qualification. Concerning the formula for the artificial anthrax fatty antigen as published, it is understood of course that it represents antigen complex made from the purest fatty acids available to, and compounded by the writer. Those who have worked much with fats will appreciate that a label does not guarantee a pure article, and that it is often a difficult matter to obtain different lots of fatty acids all having the same values. For instance, a sample of sodium oleate (Kahlbaum) had to be discarded because the liberated fatty acid gave an iodine value of 66 instead of approximating the theoretical value of 90.07; and again, a quantity of capric acid was found to consist of a mixture of caprylic and lauric acids. The oleic acid employed had an iodine value of 87.5, but when for this acid there was substituted one having a value of 80, the antigenic properties of the mixture became somewhat impaired. It goes without saying, therefore, that were the work to be repeated by the author with entirely different lots of fatty acids there would probably occur a greater or less deviation from the antigen formula given.

The reaction of agglutination-precipitation should be regarded, like all new reactions, as tentative and in the formative stage only. The word precipitation should not be construed as having any relation to "precipitin," but rather as expressing merely the settling at the bottom of the coarse agglutinated antigen-antibody aggregate. The same factors which give rise to the specific agglutination of bacteria by antibody (agglutinins) operate in the agglutination-precipitation test. The antibody recognizes and unites specifically with the antigen whether the antigen be found at the surfaces of the bacterial cells or on the surfaces of the cholesterol in the artificial antigen emulsions, these antigenic emulsions being in a sense electro-negative suspension colloids of artificial bacteria, consisting as they do of a framework of cholesterol representing a stroma or cell surface on which is adsorbed the antigen complex.

Many of the antigen emulsions in salt solution retain the colloidal state for considerable periods of time, while others precipitate spontaneously in a short time. In the latter cases it is necessary to stabilize the emulsions in such a manner that the control tubes will not flocculate and precipitate spontaneously during the time the tubes are in the ice-box. This stability can be had either by adding a trace of purified

lecithin to the alcoholic antigen solution, or by making up the salt solution in $\frac{1}{5000}$ N. NaOH solution. These substances do not impair the antigen, but tend to improve it by retarding the action of the electrolyte and the tendency to undergo hydrolysis which some complexes of alkali salts of fatty acids show sooner than others. Normal serum is of itself an excellent stabilizer.

It is an error to characterize the antigen emulsions as soap solutions, since they are not, in the first place, solutions in the strict sense of the term, and in the second place, not all alkali salts of fatty acids possess the characters of soap. Cholesterol or its equivalent is a constituent of nearly all cells and thus far has proved the best substance for the formation of particulate surfaces on which to adsorb antigenic material. It is superior to agar, starch, mastic and other higher alcohols of similar composition.

A working hypothesis for the mechanism of the reactions of immunity in the light of the facts observed during the work on antigens will form the subject of a subsequent paper.

The antigen of *B. anthracis*, like the antigens of many other bacteria, consists of a pure fatty complex particular and specific for that micro-organism, differing in composition from those of other bacteria studied.

The guinea-pigs and rabbits in this laboratory injected with large doses of the sterile artificial anthrax antigen, regularly develop anthrax infection, by a process of probable specific lowering of resistance, from anthrax spores of low virulence ingested with the baled hay. Small doses of the antigen raised the resistance of the animals against infection with a highly virulent strain of anthrax organisms.

Anthrax protein is not essential to the reactions and mechanism of immunity.

THE PRODUCTION OF ANTISERUM FOR AGGLUTINATION TESTS

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On subcutaneous and intraperitoneal inoculation of organisms, washed from agar slants and killed by heating, death of the rabbits often occurred, especially in the cases of *B. enteritidis* (Gärtner), paracolon and paratyphoid bacilli, and when the animal survived, repeated inoculations were required to produce a serum of satisfactory titer. The marked toxicity of the killed suspensions led us to try to overcome this troublesome feature, and the results are given briefly.

Growths from agar cultures were treated as before, the suspensions being killed in a water bath at 60 C. for 30 minutes, after which that portion of the tube above the liquid was flamed in order to destroy any organisms which might have survived, as the tube was not totally submerged. Cultures were then made to ascertain definitely the absence of living organisms.

TABLE 1
RESULT OF INOCULATION OF RABBITS

Rabbits	Suspension	Inoculations	Amounts in C C	Dates Killed	Final Titers
61-A	<i>B. enteritidis</i> 1	3/11	1.5		
		3/26	2	4/10	1:10,000
10	<i>B. paratyphosus</i> "A"	4/23	2	5/ 9	1:10,000
760	<i>B. enteritidis</i> 1	3/20	2	4/10	1:6,600
756	<i>B. paracolon</i>	4/14	1	4/29	1:6,600
421	<i>B. paracolon</i>	4/14	1		
		4/28	2	5/12	1:10,000
5	<i>B. enteritidis</i> 1	4/23	2		
		5/ 1	2	5/15	1:10,000
6	<i>B. enteritidis</i> 1	4/23	3		
		5/ 1	2	5/15	1:5,000
7	<i>B. enteritidis</i> 104-B	4/23	2		
		5/ 1	2	5/15	1:5,000

The following day the suspensions received at least three washings in sterile salt solution; the supernatant fluid being poured off and replaced each time with fresh salt solution; the clumps were disintegrated and the organisms distributed evenly throughout the suspensions by agitating the tube. After the third washing, salt solution was added and the suspensions filtered through cotton, the density being regulated by comparison with a nephelometer. These suspensions were inoculated in the ear vein under antiseptic precautions. The animals showed no apparent discomfort and the table illustrates the efficacy of this cell-washing method. In one instance, the first injection produced a titer of 1 to 10,000, and the results as a whole are far better than those of the older method.

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The bacterial suspensions used were washed from a 24-hour agar slant with carbolized (0.5%) salt solution, filtered through cotton and standardized by a nephelometer of such a density that the graduations on a 5 cc pipet held in the middle of a test tube of the fluid are just visible. The immune serum was made up in dilutions of 1:10, 1:100 and 1:1,000, with salt solution, and varying quantities used to give the desired range of concentrations in the test. In addition to the serum each tube contained 0.25 cc of salt solution and 0.2 cc of the bacterial suspension. The mixtures were incubated at 37.5 C. in a water bath for 24 hours and read. In positive mixtures there was a clear fluid and a characteristic "scattering" sediment with an irregular border, markedly different from the negative tubes even before the tubes were agitated to reveal the clumping.

It appears that washing rids the suspensions of certain toxic materials and facilitates the production of antiserum. It is possible that the method may be of advantage in the case of other organisms as well and in the fact that more marked agglutination is produced than when larger quantities are used.

SERUM REACTIONS IN INFLUENZA *

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There is apparently little information on the serology of influenza which is of certain etiologic or diagnostic importance. Cantani,¹ Vagedes,² Fichtner³ and Ghedini⁴ have described a few positive agglutination reactions with serum of acute and convalescent influenza cases and Pfeiffer's bacillus, but several of them, notably Fichtner, have also mentioned positive reactions, although usually in lower dilutions, with normal serum, so that the specificity of the reaction is questionable. Odaira⁵ noted a tendency to spontaneous clumping of influenza bacilli in normal salt solution. The observation of Vagedes that he obtained better results when the tests were incubated at 37 C. for from 20 to 24 hours is suggestive in view of our results at a higher temperature.

We find no reference to the use of other immunity reactions in influenza cases.

Our experiments herein briefly reported are at least suggestive from the standpoint of etiology and diagnosis, and would seem to be self-controlled, owing to the groups of cases studied, although the number of normal controls is limited.

RABBIT IMMUNE SERUM

Several rabbits were immunized by from 6 to 8 injections, the majority intravenously, but a few subcutaneously, of a mixed vaccine comprised of equal parts of 10 different strains of *B. influenzae*. These strains were originally isolated in the laboratory of Dr. W. H. Park during Sept., 1918, and had been derived from the nasopharynx or lung of cases of influenza. They were kindly placed at our disposal by Dr. G. H. Smith, of the Yale Medical School, for the purpose of this investigation. These strains were uniformly grown on a "chocolate" medium prepared by adding 5% of defibrinated rabbit or human

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¹ Ztschr. f. Hyg. u. Infektionskr., 1903, 42, p. 505.

² Deutsch. militärärztl. Ztschr., 1903, 32, p. 236. Abstract in Baumgarten's Jahresbericht über die path. Mikroorganismen, 1903, 19, p. 244.

³ Deutsche militärärztl. Ztschr., 1906, 35, p. 337. Abstract in Baumgarten's Jahresbericht, 1906, 22, p. 207.

⁴ Abst. in Centralbl. f. Bakt., 1 Abt., Ref. 1908, 42, p. 177.

⁵ Centralbl. f. Bakteriologie. I. O., 1912, 61, p. 289.

blood to 5% glycerol agar at a temperature of 95 C. On this medium all the cultures with which we have dealt grow very profusely, so that in 24 hours the Blake bottle is covered with a uniform creamy layer. Suspensions from these cultures are readily made by rocking the bottles with salt solution. They usually sediment very slowly when prepared in this manner, and do not agglutinate spontaneously with normal salt solutions, as has apparently often been the case when cultures from a plain blood medium are employed.

Our rabbits received injections beginning with 0.5 cc of a thick suspension of the mixed cultures killed by the addition of 0.5% phenol at first. On subsequent injections living cultures were employed. It was found that injections could be given at short intervals without producing any symptoms in the rabbits except slight loss in weight. The serum from these animals was tested after 6 or 8 injections.

Negative Tests.—The following reactions were tried with the serum of the immunized animals, with completely negative results:

1. Agglutination tests with dilutions of the serum of from 1:10 to 1:160 in a total volume of 1 cc, by adding three drops of a thick suspension of phenolated mixed culture as described. Several of the separate cultures used to immunize animals were also tried, and also with negative results. In these experiments the tubes were incubated at 37 C. for 2 hours and then allowed to stand at room temperature for 18 hours.

2. Pfeiffer's phenomena of bacteriolysis (Cantani).

3. The conglutination reaction (Bordet and Gay).

4. Precipitin tests with an antigen obtained by grinding the vaccine of mixed influenza strains precipitated and dried by alcohol and ether, ground, extracted with phenolated salt solution, and centrifugalizing until the supernatant fluid was perfectly clear.

Positive Reactions with Rabbit Immune Serum. Fixation Reactions.—These reactions were carried out with the immune serum and also with the serum of patients, as will be described later, with the following method: Tubes containing 0.1 cc or less of the serum to be tested with and without the antigen. The antigen employed in this experiment consisted of a mixture of the 10 influenza strains, centrifugalized, killed and precipitated with absolute alcohol and absolute ether, and dried over calcium chlorid. This dried vaccine was then ground in an agate mortar and suspended in 0.5% phenolated salt solution in the proportions of 1 mg. of dried bacteria to 1 cc; $2\frac{1}{2}$ units of fresh guinea-pig serum were added to the combination of antigen and serum, and one half of the inhibiting dose of antigen with a control normal serum was employed. After incubation for one-half hour at 37 C., 0.5 cc of washed sheep blood was added, plus 2 hemolytic units of rabbits antiserum. The reactions were observed after one-half hour's incubation at 37 C., and on standing at room temperature until the following day.

The serum of immune rabbits was found to give a complete inhibition of hemolysis, that is, complete fixation, in a dose which in one instance was as low as $\frac{1}{320}$ of a cubic centimeter.

Agglutination Reactions at 55 C.—The rabbit immune serum gave negative results in agglutination tests incubated at 37 C. for 2 hours, as already stated. The observations of Vagedes, and particularly a personal communication from Major Leslie H. Spooner, led us to try the effect of longer incubation at body temperature. This method gave positive results with the rabbit immune serum.

Further experiments showed that the best results are procured by incubating at 55 C., as is the case with meningococcus agglutinations. Clumping and complete sedimentation in from 3 to 6 hours was found with the serum of different immune rabbits in dilutions of from 1:100 to 1:800, while normal serum controls remain negative at 1:50. There is some evidence that these agglutinins are quickly lost, as an unheated serum of high titer tested at intervals within a month dropped from 1:800 to 1:50.

There was also a variation in the agglutinability of different strains of *B. influenzae* which strongly suggests the existence of different groups or varieties of influenza bacilli. Our successful results both with rabbit immune and human serum have been with one of the strains used in immunizing the rabbits (strain C from nasopharynx, Sept. 5, 1918). Two other strains of *B. influenzae* were also tried with the rabbit serum. The one strain (strain L, lung, Sept. 21, 1918) had not been used in the mixed vaccine with which the animals were immunized. The other strain had been derived from the nasopharynx of an acute case of influenza within 10 days. The serum failed to agglutinate these strains in a dilution of 1:50.

SERUM REACTIONS WITH INFLUENZA CASES

Serum from acute cases of influenza; from recovered cases of influenza; from persons vaccinated by means of three injections, totalling three thousand million organisms, of a mixed vaccine prepared from eighteen Park strains, and from a few normal cases have been tested.

Negative Reactions.—The following reactions gave completely negative results when tested with serum representing the categories just mentioned:

1. Agglutination reactions with short incubation at 37 C., in accordance with the usual method, as previously outlined.
2. Conglutination reactions.
3. Opsonic tests.
4. Precipitin tests.
5. Intradermal injections of alcohol-killed, ground cultures of *B. influenzae* in a series of vaccinated and recovered cases.
6. Fixation tests, using as antigen the sputums of influenza cases prepared after the method of Krumwiede and Valentine,⁶ using both the serum of the patient from whom the sputum was derived, and of several other acute cases.
7. Fixation tests, using the urine as an antigen, prepared after the method described by Dochez and Avery⁷ for the precipitin tests in pneumonia, both with the serum of the individual patient and other patients as antibodies.
8. Fixation tests were tried with two separate antigens, prepared in the manner described, from gram-negative diplococci that were frequently found in the bacterial flora of the acute cases.

Positive Serum Reactions.—Fixation reactions were obtained in 40% of twenty-five vaccinated cases, and in only one of twenty-nine acute cases of influenza. They were negative in normal individuals and in the recovered cases.

Agglutination reactions with phenolated suspensions of influenza bacilli with short incubation at 37 C. were negative, as in the case of the rabbit immune serum. Definite positive reactions were at once found on incubation at 55 C. with some of the serum, while others remained negative. In from 3 to 6 hours large mucoid floccules appear in the positive tubes which promptly settle in a

⁶ Jour. Am. Med. Assn., 1918, 70, p. 513.

⁷ Jour. Exper. Med., 1917, 26, p. 477.

compact mass at the bottom. In the negative tubes the suspension of bacteria remained homogeneous. If the tubes were removed from the 55 C. bath at the end of 6 hours and allowed to stand over night at room temperature, the readings are the same on the following day, in the majority of instances. With some of the phenolated suspensions, however, the differentiation between serum that at 6 hours are distinct may not be clear on the following day. We must confess that we have been led to frequent repetitions of these experiments through some uncertainty as to the correct criterion of differentiation. The results at 6-hour reading are sharp, and owing to the considerable number of serums from different groups of cases tested, would seem to be self-controlled. Nearly 90% of the acute cases of influenza gave positive agglutination reactions in a dilution of from 1:40 to 1:160, whereas all marked as negative failed in a dilution of 1:10. The serum of previously vaccinated individuals were positive in 45%, and recovered cases in 38.5%, apparently irrespective of the time after recovery. Very few normal serums were available. It is also unfortunate that the serums of other acute febrile conditions as a control on the positive reactions were not available.

TABLE 1
FIXATION REACTIONS WITH MIXED INFLUENZA ANTIGEN AND HUMAN SERUM

	No. Tested	Positive	Percentage
Acute cases of influenza	29	1	3
Recovered cases (from 10 to 48 days after normal temperature)	17	0	0
Vaccinated (from 2 to 37 days after last injection).....	25	10	40
Normal serum	5	0	0

TABLE 2
AGGLUTINATION REACTIONS AT 55 C. WITH CULTURE C

	Number Tested	Positive	Percentage
Acute cases	25	22	88.0
Vaccinated	24	11	45.8
Recovered	13	5	38.5
Normal serum	9	0	0.0

It should be added that the results noted were obtained with a phenolated suspension of culture C. Reactions with culture L were uniformly negative. A few positive reactions were obtained with the serum of acute cases and culture 69, but most of them were negative, as were the other serums studied.

SUMMARY

The serum of rabbits immunized by means of a suspension of mixed cultures of *B. influenzae* gave for the most part negative immunity reactions. Fixation antibodies were found in high dilutions, using a preparation of the same mixed cultures as antigen. Agglutination reactions were negative when incubated for a short period at 37 C., but positive in from 3 to 6 hours at 55 C. The immune serum failed to react with two strains of *B. influenzae* that had not been used in immunizing, thus suggesting the existence of separate groups of *B. influenzae*.

The serum of acute influenza cases gave a positive agglutination reaction at 55 C. in dilutions of 1:80 and above, in the majority of instances (88%) with one of the strains of *B. influenzae* tested. A few tests with another recently isolated strain from our epidemic were also positive in a few instances. Another strain was uniformly negative. Fixation reactions were negative in all but one case with a mixed antigen.

The serum from individuals that had recovered from influenza gave agglutination reactions at 55 C. in about two thirds of the instances. Fixation reactions were negative.

The serum of individuals that had been vaccinated gave agglutinations at 55 C. in 45% of the cases. Fixation reactions were positive in 40%.

The normal cases, although few in number, were uniformly negative.

SOME EXPERIMENTS ON THE TRANSMISSION OF INFLUENZA *

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The prevalent vague and contradictory conceptions of influenza, its etiologic agent and its mode of transmission are responsible for the absence of uniform and systematic measures for its control and the comparative helplessness of the medical profession in the face of its ravages. Animal experimentation does not lend itself to the use of material or organisms isolated from influenza patients, making the use of human subjects for experiment the best hope for a rational basis for the control of the disease.

While Pfeiffer's bacillus is frequently found in the mucous membrane of the respiratory tract and in the lungs of influenza patients, its relation to the etiology of this disease has not been established, especially in view of the fact that other organisms, such as the pneumococcus, diplococcus, pleomorphic streptococcus, micrococcus catarrhalis, etc., have been reported frequently not only in association with, but also in the absence of, the influenza bacillus. On the other hand, the recent communications of Nicolle and others,¹ brief and inconclusive as their experiments are, lends color at least to the view that the exciting cause may be a filtrable virus, and that the influenza bacillus bears but a symbiotic relationship to it, analogous to *B. aertrycke* and the virus of hog cholera.

The present investigation was made for the purpose of ascertaining some possible factors in the mode of transmission of influenza through the use of human subjects for experiments. Two objectives were sought: first, to determine the infectious nature of bacteria free-filtrates as reported by Nicolle; second, to test the pathogenicity of

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* The observations recorded in this paper are a portion of a report submitted by a board appointed by Colonel C. F. Craig, commanding officer, Yale Army Laboratory School, New Haven, Conn., to investigate the etiology of influenza. Authorization for the human experimentation that was necessary was granted by the Surgeon-General who authorized the board to call for volunteers for the study of influenza virus. Six officers of the Sanitary Corps of the army responded and the nature of the experiment was explained to each of these officers, together with the risk that they were taking in submitting themselves for inoculation with material supposed to contain the virus, and they all signified their willingness to serve and the result of the experiments is given in this paper.

¹ Compt. rend. Acad. d. sc., 1918, 167, p. 607; Dugarrie de la Riviere, R.: *ibid.*, p. 606.

various typical strains of influenza bacilli for man, and ascertain if their use will result in the production of the disease.

The plan and course of the investigation were:

A. Observation of the Volunteers.—Six apparently normal men (officers assigned to the school), having been fully informed of the possible hazards, volunteered as subjects. They were quartered and strictly isolated in the isolation pavillion of the hospital. Two of the volunteers had not been vaccinated with any influenza vaccine (volunteers 4 and 5), while the others had taken a polyvalent influenza vaccine from 4-6 weeks previously. Volunteer 5 had, however, had pneumococcus vaccine. None had had influenza.

On admission to the hospital all of the men were kept under observation for 4 or more days before being used. Careful physical, clinical and laboratory examinations were made and recorded during this period. Two sergeants were detailed to attend to the wants of these men. Blood was taken for serologic study by Majors Gay and Harris. No antibodies against the influenza bacillus were found.

During the period of observation the bacterial flora of the nares and the nasopharyngeal vault of each volunteer were studied. In swabbing, care was exercised to secure the secretions of the nose and nasopharynx free from incidental contamination from the nostrils or buccal cavity. The material on the swabs was then immediately streaked on plates of (a) whole-blood agar, (b) chocolate agar of Williams,² and (c) Avery's oleate hemoglobin agar.³

Preparation of Medium.—(a) Blood agar: To every 100 cc neutral beef infusion, 5 cc fresh sterile defibrinated sheep blood was added at 45-50 F., and after thorough mixing poured in the plates.

(b) Chocolate agar: 3 cc fresh defibrinated sheep blood was added to every 100 cc of neutral beef infusion agar containing 5% glycerol. It is important that the blood be added while the glycerol agar is hot; that is, immediately after the latter is taken from the Arnold sterilizer (95 F.). The medium should have a dark chocolate color. If light in color it does not work as well.

(c) Sodium oleate hemoglobin agar (Avery): Add the washed cells of 1 cc fresh sterile sheep blood and 5 cc of a sterile 2% watery solution of neutral sodium oleate to 95 cc of hot (95 C.) 2% infusion agar, neutral to — 0.3% reaction.

In each case the melted medium was cooled to 42 F. before pouring in order to reduce the water of condensation to a minimum. All plates were incubated from 24-48 hours before use. Cultures and plates were held in the incubator (37 C.) and reexamined daily. Colonies were described in the order of their predominance, smears made and examined and subcultures made on chocolate, blood, oleate and infusion-agar plates.

During the period of preliminary observation one of the volunteers (4) developed an acute catarrhal infection of the upper respiratory tract which made it advisable to exclude him from the investigation, even though the symptoms, course and blood picture were not characteristic of influenza. Because of this occurrence the experiments on the other volunteers were postponed four days.

B. Experiments.—*Exper. 1.* The purpose of this experiment was to determine the infectiousness of the filtrates of typical influenza lungs when applied

² Park and Williams: Pathogenic Micro-Organism, 1917, p. 103.

³ Jour. Am. Med. Assn., 1918, 71, p. 2050.

to the nasopharyngeal mucous membrane of man. Two filtrates were prepared; one (152) was obtained from the pneumonic area of a lung from a typical case of influenza pneumonia. The tissue was obtained 4 hours after death. The organisms isolated from the lung were (a) pneumococcus-type 2 (from blood, pleural fluid and lung tissue). (b) *B. mucosus*, and (c) *M. catarrhalis*. No influenza bacilli were identified from the respiratory tract of this case. The other filtrate was taken from the pneumonic area of a typical case (154) of influenza. Influenza bacilli were isolated from this case. In addition a streptococcus viridans, a pneumococcus type 4, and a staphylococcus albus were identified as being present. Filtrates of the sputum were not made because of the lack of suitable material. The material from 154 was obtained about 24 hours after death.

Ten grams of the tissue were finely chopped, ground up with salt solution and sterile sand in a mortar, centrifuged and the supernatant fluid passed through a Berkefeld filter and the clear filtrate collected aseptically. Bacteriologic tests showed it to be sterile.

The filtrate was placed in a DeVilbiss atomizer and sprayed heavily in each nostril and the nasopharynx of two of the volunteers (1 and 3); three squeezes of the atomizer bulb were given in each location. The atomizers were tested as to their efficiency and were in good order.

No apparent clinical effect followed the use of the filtrates. No subjective symptoms were noted. The temperature curve was unchanged and the blood picture presented nothing unusual. Daily bacteriologic examinations of the nares and pharyngeal vault both before and after the use of the filtrates showed no noteworthy change either in the way of suppression or stimulation of former types or the appearance of new types of bacteria.

Expt. 2. This consisted in applying various strains of *B. influenzae* to the nasopharyngeal mucous membrane in order to determine their pathogenicity.

The following typical strains of the influenza bacillus were used: Strain C obtained through the kindness of Dr. George Smith from Dr. William H. Park, isolated in September, 1918, from nasopharynx. Strain L from the same source, Sept. 21, 1918. Strain 69, isolated at this laboratory on Nov. 26, 1918, from the nasopharyngeal secretion at the height of the disease and 24 hours before the death of the patient. This organism was associated with an indifferent streptococcus and *M. catarrhalis*. This strain was 15 days old when used.

Every precaution was taken that the above strains were pure before use. They were plated, single colonies fished, stained and planted on blood, chocolate, oleate and plain infusion-agar plates. The morphology was typical of Pfeiffer's organism. The profuse 24-hour growth on one plate of chocolate-blood agar was thoroughly emulsified in salt solution and the volume made up to 10 cc, in each case, giving a heavy milky appearing suspension. A loopful of this suspension was spread out on chocolate, oleate and plain infusion-agar plates. In each case typical profuse growths occurred on the first two but no growth on the last.

All (5) of the volunteers were used. The normal bacterial flora were determined by almost daily examinations of the nares and nasopharyngeal vault for a period of from 4-7 days prior to this experiment. As soon as the suspension of the influenza bacilli was made and control plates planted, each was placed in a sterile DeVilbiss atomizer and immediately sprayed into each nares and, through the mouth, into the nasopharynx of the proper volunteer by three complete compressions of the atomizer bulb. In each case the

atomizer was tested to see if it was in good order and a heavy spray issued each time. The nares and nasopharynx were swabbed and the material plated just before giving the spray. Strain C was given to volunteers 1 and 6, both inoculated against influenza about 6 weeks ago; but the blood showed no antibodies against Pfeiffer's organism. Strain L was given to volunteer 2. Strain 69 was given to volunteers 3 and 5 (5 being the only one who had not been inoculated with an influenza vaccine).

Culture tests on the atomizer after use showed that *B. influenzae* was present, viable and in pure culture.

In the next few days no untoward symptoms, abnormal physical or clinical findings were observed. The temperature curve presented its usual normal range. There was no distinct change in the blood picture. In fact, there were no symptoms in the least suggestive of influenza.

On the first day bacteriologic examinations of the nose and throat were made every 4 hours, twice the next day and daily thereafter. There was no material change in the bacterial flora except the addition of the influenza colonies which appeared in all of the plates the first two days after spraying, but then disappeared quite rapidly from the nares, though persisting in the nasopharynx 1 to 3 weeks or even longer, and this even in spite of the use of dichloramin T.

Exper. 3. This was made to determine the pathogenicity of a freshly isolated strain of the influenza bacillus, strain 162, from the unconsolidated portion of the lung of a fatal case of influenza pneumonia in which the influenza bacillus was present in large numbers along with an indifferent streptococcus, staphylococci of both albus and aureus types and a gram-negative bacillus belonging to the Friedländer type. The patient died at 10:30 a. m., and a necropsy was held at 3 p. m. of the same day when the cultures were made. The following morning the chocolate and oleate and blood agar showed many typical influenza colonies. Discrete colonies were fished and spread on oleate and chocolate plates. The following morning the plates showed a pure culture of what were apparently influenza bacilli. A suspension was made from the profuse growth on the chocolate plate and used precisely as in the second experiment. Controls of the contents of the atomizer both before and after use showed that the material used contained viable influenza bacilli in pure culture. Subsequent study proved this strain to be a typical strain of *B. influenzae*.

The suspension was made and introduced in the volunteers in the same way as in the previous experiment and was given to volunteers 5 and 6 (four days after they had a previous spray of influenza bacilli in experiment 2). This suspension represented the second generation of a strain of influenza bacilli only 43 hours removed from the infected lung.

The temperature curve showed no striking change; number 6 showed a slight rise (0.5 F.) on the afternoon of the same day, and he said he felt as if he had a slight cold in the head, but this disappeared the following day. The rest of the clinical course was uneventful and nothing suggestive of influenza appeared. The bacteriologic findings were identical with those following the second experiment.

The bacterial flora of the nose and throat was studied in detail in order to detect organisms, such as hemolytic streptococci, bacilli of the Friedländer type, pneumococci, *M. catarrhalis*, etc., that might contribute to complications and particularly for the purpose of determining or excluding a possible symbiotic rôle of any of the bacterial types found.

As stated, the bacterial flora was fairly constant for each individual and remained unchanged after the use of either the filtrate or the suspension of living bacilli, with the exception of the addition of a greater or less number of influenza colonies after the spraying in of these organisms.

The second point is the persistence of influenza bacilli in the nasopharynx as contrasted with their early disappearance from the nose. In the latter (except in 5) they disappeared in less than 72 hours, while in the throat they were present several weeks, and in one case present at the time of writing (1). It may be that staphylococci and diphtheroids, more common in the nose than in the throat, may inhibit the growth of influenza bacilli and be responsible for their early disappearance, whereas the types found in the throat are not so antagonistic. As might be anticipated, more types of bacteria were present in the nasopharynx than in the nares. In the former streptococci of the viridans and indifferent types were almost constantly found, while in the latter they were either absent or when present, found in relatively small numbers. The absence of hemolytic streptococci, pneumococci and bacilli of the Friedländer type is significant in view of the negative findings in this investigation, and their frequent occurrence in influenza patients.

The appearance and persistence of the influenza bacilli after their experimental introduction in the throat is of considerable interest. Not only was the organism present in the nasopharynx of all of the volunteers at the end of 12 days, but it was present in considerable numbers. It is also noteworthy that after 3 or 4 days the organisms became relatively scanty for a day or two and then reappeared in much larger numbers, and even as the predominating organism. This suggests that the mucous membrane of the nasopharynx of man is a favorable habitat for this organism, and that the bacillus not only exists, but multiplies there.

The bacterial flora of volunteer 6 deserves special consideration. In every culture of his nose and throat throughout his observation a small gram-negative bacillus was present which in every respect (except in the form of some of its colonies) would be classified as a strain of the influenza bacillus. It grew more luxuriantly than the other strains used and the moist confluent appearance of its colonies suggested more those of bacilli of the Friedländer group. Yet their consistency was not the same and seemed more like that of *B. influ-*

enxae. The colonies were not nucleated, and this aided in differentiating them from those which were introduced and which were nucleated colonies. These bacilli consistently failed to grow on plain infusion agar, serum agar, on Loeffler's coagulated serum, or in plain infusion broth. It grew only on mediums containing blood. At times the morphology was confusing because of the pleomorphism it showed. At one time smears from discrete colonies contained only sharply defined, intensely staining bacilli with thread forms, and the majority of the individuals were larger or more sharply defined than was ever observed in the typical strains of *B. influenzae*. Yet frequently, especially on the chocolate plates, these bacilli had the classical morphology of the influenza bacillus, not only in the short, delicate, slender bacillary and coccil forms, but also in their faintly staining properties. This organism was nonpathogenic for rabbits when given in large doses either intravenously or subcutaneously, which was also true of the four strains of *B. influenzae* implanted in the nasopharynx of the volunteers. The organism was therefore tentatively classed as an influenza bacillus. Although this man had never had influenza, he had been exposed in the recent epidemic and was also in the sudden transient epidemic of "grippe" at Fort Oglethorpe in the spring of 1918.

Ten days after exper. 2 was begun, none of the volunteers showing any symptoms, but all showing more or less heavy growth of influenza bacilli from the nasopharyngeal secretion, volunteers 1, 2 and 3 were treated twice daily with a spray of dichloramin-T in chlorinated eucalyptol, and 2 days later the other two volunteers were treated in the same way in an effort to clear their throats. A few days later they were discharged from the hospital, but influenza bacilli were still present and even in greater numbers at times, although the number and types of other organisms were appreciably diminished, in some instances to such an extent that the influenza bacilli were present apparently in pure culture. This persistence of the influenza bacillus in the presence of such a powerful disinfecting substance as dichloramin-T is in accord with the difficulty some clinicians have noted in ridding convalescent influenza patients of this organism.

Following his discharge, one of the volunteers (6) used chlorazene for several days, resulting in the disappearance of the influenza bacilli. Three of the volunteers subsequently presented negative cultures, but one (1) still showed many colonies of typical influenza bacilli.

In all of the experiments the general condition of the men was apparently unaffected by the application of bacterial free filtrates of influenza material, or by the suspension of the various strains of influenza bacilli. In no case was there the least symptoms of influenza. The white blood cell count tended to be low, but not different from the period before the inoculation. The differential count showed nothing unusual.

The freshly isolated strain produced no more effect than the older strains. The filtrate experiment cannot be considered conclusive because it does not include enough cases; the filtrate was too old and material should have been taken earlier in the course of the disease, when the virus, if such is the case, would be more virulent. Some doubt is cast on the conclusions reached by Nicolle as to the existence of a virus.

The fact that the volunteers received such massive doses of influenza bacilli, much larger than could possibly happen through natural means, with no symptoms, makes it very doubtful if this organism alone can cause this widespread epidemic. To be sure, the fact that some of the volunteers were vaccinated against influenza vitiates the value of these negative results somewhat, yet 5 who had not been vaccinated, behaved in the same way and then, too, the efficacy and protective power of the vaccine is very much in doubt. Culture 69 was taken from a fatal case of influenza, the patient receiving the same vaccine that the volunteers did, about a month before his death.

CONCLUSIONS

The nasal application of a filtrate from a pneumonic lung of an individual dead from typical influenza bronchopneumonia failed to call forth any abnormal symptoms.

The application to the mucous membrane of the nares and nasopharynx of five healthy men (four inoculated from 4-6 weeks ago against influenza with a polyvalent influenza vaccine, one uninoculated) of freshly prepared suspension of four different live strains of *B. influenzae* (one, in the second generation from the fatally infected human host) even in the massive doses, failed to produce any abnormal symptoms.

The implantation of living suspensions of influenza bacilli produced no material alteration besides the addition of the influenza bacillus itself.

When experimentally implanted the influenza bacillus disappears from the nares in a relatively short time, from 24-72 hours.

When experimentally introduced in the nasopharynx of man the influenza bacillus exists and multiplies for a considerable length of time, two weeks or more, and apparently shows considerable resistance to the action of dichloramin T.

In the examination of the nasopharyngeal secretions of patients suffering from infections diagnosed clinically as typical influenza and in tissues of the respiratory tract of patients who died of influenzal bronchopneumonia, it was found that the oleate plates frequently gave positive results when the blood and chocolate plates were negative and in addition, by inhibiting the growth of streptococci and pneumococci, greatly facilitated the isolation of the influenza bacillus. In every case the chocolate plate gave more information than the blood plate, which was useful only in picking pneumococci and making a hasty diagnosis of the type of streptococcus present.

It is recommended that in the routine bacteriologic examination of all suspected influenza cases, plain, infusion, chocolate and oleate or soap agar be used. The blood plate should not be discarded, because it gives information regarding the presence or absence of streptococci, and pneumococci, that are liable to be missed otherwise and that may play a not unimportant part either in a symbiotic rôle or as a complicating factor.

THE INFLUENCE OF DESICCATION ON CERTAIN NORMAL IMMUNE BODIES

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Many methods of drying have been applied to serum, vaccines and other substances for the purpose of preserving conveniently for a considerable length of time, under variations in temperature, the essential activities of such products. The practical objections to the results thus obtained are too great a loss of titer and the difficulty of solution.

Shakell¹ claims to have overcome these objections to a certain degree. By freezing liver and guinea-pig serum, while desiccation was going on, over sulphuric acid in vacuo, hydrolysis was prevented, the diastatic ferment of the liver and the complement of the serum were preserved. On dissolving the dried serum a clear solution resulted. Harris² applying this method to rabid brains, demonstrated activity after 4 months, the virus retaining from $\frac{1}{3}$ to $\frac{1}{2}$ its original virulence. Van Steenberge³ was able to maintain virulence for several months by spreading brain emulsion in thin layers on porcelain plates and desiccating very rapidly in vacuo. Marie⁴ repeated these experiments with some success. Shakell and Harris⁵ believe that the thinly spread material is frozen by the rapid evacuation, that freezing maintains the particles in their normal relations so that the concentration of salts and other substances, which at ordinary temperature are in solution, is prevented. Drying, they believe, proceeds from the surface from cell to cell.

In our opinion this explanation does not seem adequate. It is quite possible that the spreading and rapid drying facilitates the cell to cell drying, while the cold may play an entirely different rôle by checking processes of a fermentative nature. In our experiments, serum dried in the upper part of the desiccator which was not immersed in the freezing mixture, but evacuated under the same conditions, showed a lower agglutinating index than the serum that was frozen.

Achalme's⁶ work with smallpox vaccine helps to bear out this point. He states that the action of glycerol is not directly bactericidal, but by extracting the soluble ferments from the cells sets free an agent which is the destructive element. He maintains further that glycerol added to the crude material acts better than when added to the finely triturated substance, as in the latter case the ferments are set free more completely, and being in excess attack not only the bacteria, but the vaccinal agents as well.

Burrows and Cohn,⁷ in a quantitative study of the evaporation of blood serum, found that prolonged warming attendant on drying in a current of air

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¹ Jour. Physiol., 1909, 24, 325.

² Jour. Am. Pub. Health Assn., 1911, 7, 52.

³ Compt. rend. Soc. de Biol., 1903, 55, 1046.

⁴ Quoted by Shakell and Harris, 5, 1.

⁵ Shakell and Harris, Jour. Infect. Dis., 1911, 8, 47.

⁶ Bull. de la Soc. de Path. exotique, 1909, 2, 431.

⁷ Jour. Biol. Chem., 1918, 36, 587.

at atmospheric pressure tended to denature the proteins. They resorted to drying under reduced pressure in the presence of calcium chlorid at a temperature between 45 and 50 C. They observed that the best results were obtained if the serum was allowed to drop slowly on the sides of the flask so timed as to make the rate of evaporation and the addition of fresh serum correspond. They claim that serum so dried is practically free from electrolytes, and is faintly alkaline, the P_H being 8.1, while the original serum was 7.6. Redissolved in its original concentration the reaction of the dried serum was P_H 0.4.

Most of the work reported in connection with immune serum has been with serum of high titer, in which even fairly high percentage losses of titer would still leave the serum with satisfactory properties. Little work has been done with serum containing normal immune bodies, usually of low titer, in which considerable percentage losses would make a great difference in their practical use. The following experiments were conducted for the purpose of making a comparative study of the effects of the different methods of drying on the normal antibodies in the blood of different animals.

It was found that the blood of the normal horse and normal goat contained agglutinins for *B. dysenteriae*, Flexner type, and hemagglutinins for rabbit cells in sufficient quantity for a practical working basis.

The preparations were made by spreading 0.3 cc of serum on slides or in flat-bottomed glass dishes. The following methods of drying were followed: drying in the air or in vacuo over sulphuric acid at room temperature, and freezing and drying over sulphuric acid in vacuo. Normal horse serum was also dialyzed and treated in the same manner. Strips of filter paper were saturated with definite amounts of serum and dried in the air at room temperature. This latter method was not satisfactory on account of the excess amount of salt solution required to dissolve out the dried residue.

The dried serum was taken up by adding 0.85% salt solution up to the original amount, allowing to stand about 20 minutes, the solution and residue being thoroughly mixed and taken up. The slide was then washed off with the same amount of salt solution which was added to the first part. The clearness of the solutions differed irrespective of the method of drying employed, and did not appreciably affect the results. The frozen specimens on the whole, however, gave the clearer solutions. The freezing and desiccation over sulphuric acid in vacuo was carried out according to the method of Shakell, but it was not absolutely certain that in every instance the frozen state was maintained throughout the entire period of desiccation. The lower part of the desiccator was immersed in a freezing mixture and allowed to remain while the serum was being prepared. This cooled the dish and the air in the dish. The slides or dishes were placed on the floor of the desiccator which was turned rapidly about in the freezing mixture. A few moments sufficed to freeze the serum solidly; the sulphuric acid was then placed in the upper part of the desiccator, the cover properly adjusted and all connections sealed with a mixture of one part wax and six parts vaselin. Evacuation was produced to a pressure of from 15-40 mm. by means of a water pump. The freezing mixture was maintained at 10 C. for about 15 hours. The sulphuric acid was agitated from time to time to prevent accumulation of water on the surface. In two instances the desiccator was left in the freezing mixture for from 6 to 7 hours, then transferred to the freezing compartment of the refrigerator and allowed to remain for 3 days. There was not any apparent difference in the effects of the two methods.

The effects of desiccation on the agglutinins and hemagglutinins found in the serums of normal horse and normal goat and normal horse serum dialyzed are given in the following tables:

TABLE 1

NORMAL HORSE SERUM DRIED AND TREATED WITH *B. DYSENTERIAE* FLEXNER FOR AGGLUTININS

Number of Days Dried	Original Serum		Frozen and Dried over H_2SO_4 in Vacuo				Dried over H_2SO_4 in Vacuo				Dried in Air		
	1:400	1:500	1:50	1:100	1:200	1:300	1:50	1:100	1:200	1:300	1:100	1:200	1:300
11	+++	+	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	—
20	+++	+	+++	+++	+++	+++	+++	+++	+++	++	+++	++	—
27	+++	+	+++	+++	+++	++	+++	+++	+	—	+++	+	—
41	+++	+	+++	+++	+++	+	+++	+++	—	—	omitted	+	—
51	+++	+	+++	+++	++	—	+++	+++	—	—	omitted	—	—
59	+++	+	+++	+++	++	—	+++	+++	—	—	+++	—	—
73	+++	+	+++	++	—	—	+++	+	—	—	omitted	—	—
111	+++	+	+++	+	—	—	+++	+	—	—	omitted	—	—

+++ complete agglutination; ++ fair agglutination; + trace of agglutination; — no agglutination.

TABLE 2

NORMAL HORSE SERUM DRIED AND TESTED WITH RABBIT CELLS FOR THE HEMAGGLUTININS

Number of Days Dried	Original Serum		Frozen and Dried over H_2SO_4 in Vacuo				Dried over H_2SO_4 in Vacuo				Dried in Air			
	1:12	1:16	1:4	1:8	1:12	1:16	1:4	1:8	1:12	1:16	1:4	1:8	1:12	1:16
13	+++	++	+++	+++	++	—	+++	++	—	—	+++	++	—	—
20	+++	++	+++	+++	—	—	+++	++	—	—	+++	++	—	—
51	+++	++	+++	++	—	—	+++	+	—	—	+++	+	—	—
59	+++	++	+++	++	—	—	+++	—	—	—	+++	—	—	—
73	+++	++	+++	—	—	—	omitted	—	—	—	+++	—	—	—
111	+++	++	omitted	—	—	—	++	—	—	—	omitted	—	—	—

TABLE 3

NORMAL GOAT SERUM DRIED AND TESTED WITH *B. DYSENTERIAE*, FLEXNER, FOR AGGLUTININS

Number of Days Dried	Original Serum		Frozen and Dried over H_2SO_4 in Vacuo				Dried over H_2SO_4 in Vacuo				Dried in Air			
	1:200	1:300	1:50	1:100	1:200	1:300	1:50	1:100	1:200	1:300	1:50	1:100	1:200	1:300
15	+++	—	+++	+++	+	—	+++	+++	—	—	omitted	—	—	—
20	+++	—	+++	+++	—	—	+++	+++	—	—	+++	+++	—	—
25	+++	—	+++	+	—	—	omitted	—	—	—	omitted	—	—	—
65	+++	—	+++	—	—	—	—	—	—	—	+	—	—	—
69	+++	—	+	—	—	—	omitted	—	—	—	omitted	—	—	—

According to the tables, there is a decrease in the agglutinating power of the serum drying for 11 days both for *B. dysenteriae* and for rabbit cells. The serum that was frozen and dried over sulphuric acid in vacuo shows less loss than when dried over sulphuric acid in vacuo at room temperature, while that dried in air shows a still greater loss than the other two. The decline takes place a little more gradually with the frozen than with the nonfrozen serum, while the serum dried in the air loses its agglutinating power most rapidly of all. The ratio of loss for the various methods is fairly well maintained through the time.

Tables 3 and 4 give the results of drying normal goat serum.

TABLE 4
NORMAL GOAT SERUM DRIED AND TESTED FOR HEMAGGLUTININS WITH RABBIT CELLS

Number of Days Dried	Original Serum	Frozen and Dried over H_2SO_4 in Vacuo				Dried over H_2SO_4 in Vacuo			Dried in Air		
	1:12	1:4	1:8	1:12	1:4	1:8	1:12	1:4	1:8		
15	+++	+++	+++	+	+++	+++	—	+++	—		
20	+++	+++	+++	—	omit	ted		+++	omit	ted	
55	+++	+++	—	—	—	—		++	—		
65	+++	+++	—	—	—	—		+	—		
69	+++	+	—	—	—	—		omit	ted		

TABLE 5
NORMAL HORSE SERUM DIALYZED AND TESTED WITH B. DYSENTERIAE FOR AGGLUTININS

Number of Days Dried	Original Serum		Frozen and Dried over H_2SO_4 in Vacuo			Dried over H_2SO_4 in Vacuo			Dried in Air			
	1:200	1:300	1:100	1:200	1:300	1:100	1:200	1:300	1:50	1:100	1:200	1:300
11	+++	—	+++	+++	—	+++	+++		+++	+++	+++	—
20	+++	—	+++	+++	—	+++	+++		+++	+++	+++	—
59	+++	—	+++	—	—	+++	—		+++	+++	—	—
67	+++	—	+++	—	—	—			+	—	—	—

TABLE 6
NORMAL HORSE SERUM DIALYZED AND TESTED WITH RABBIT CELLS FOR HEMAGGLUTININS

Number of Days Dried	Original Serum		Frozen and Dried over H_2SO_4 in Vacuo			Dried over H_2SO_4 in Vacuo		Dried in Air		
	1:12	1:16	1:4	1:8	1:12	1:8	1:12	1:4	1:8	1:12
15	+++	—	+++	+++	++	+++	++	+++	+++	+
25	+++	—	+++	+++	++	+++	++	+++	+++	—
55	+++	—	+++	+	—	omit	ted	+++	—	—
65	+++	—	+++	—	—	omit	ted	+	—	—

The findings are similar to those obtained with normal horse serum. Practically the same ratio of loss for all methods holds in the case of the goat serum as in the horse serum.

Tables 5 and 6 give the results of drying normal horse serum that had been dialyzed 4 days. After this length of time the serum failed to show a trace of sodium chlorid.

It will be noted that there was a loss of agglutinins during dialyzation. No loss occurred, however, after drying under 20 days, whereas the first loss in the nondialyzed serum occurred during the first 10 days. On the 59th day the bacterial agglutinins are uniformly reduced one-half; on the 67th day the strength of the frozen serum remains the same as on the 59th day, while the serums dried in the air and over sulphuric acid in vacuo have lost the agglutinating power in the dilution of 1:50. The hemagglutinins, with the exception of a slight loss over the original serum in the first 20 days, follow very much the same course.

The retention of the agglutinin content of the dried dialyzed serum lends support to the idea of Shakell and Harris that salt concentration brings about a loss in the active elements of various substances subjected to drying, but the subsequent decline of the agglutinins after a longer period of desiccation justifies our opinion that other factors also aid in the destruction of these properties.

Consideration, however, must be given to the possibility of nondialyzable salts being present. Greenwald⁸ found that serum dialyzed for 4 or 5 days still contained about 0.3 mg. of an acid soluble phosphorus per 100 c.c.

We made some observations on the crystallization of serum. The results were sufficiently interesting to suggest future possibilities along these lines. As observed microscopically, the crystallization in the dried serum was uniform in all three methods but on dissolving in distilled water and recrystallizing by evaporation in air, a decided change took place in the formation of the crystals, certain forms being entirely absent. The dialyzed serum showed no definite crystall formation.

TABLE 7

NORMAL HORSE SERUM BACTERIAL AGGLUTININ INDEX 1:400. CELL AGGLUTININ INDEX 1:12

	Number Days Dried	Method of Drying	Bacterial Agglutinin Index	Hemag- glutinin Index	pH
Normal horse serum dried	17	Frozen H ₂ SO ₄ in vacuo.....	1:300	1:8	7.3
	17	Air.....	1:200	1:8	7.3
	51	Frozen H ₂ SO ₄ in vacuo.....	1:200	1:8	7.2
	51	H ₂ SO ₄ in vacuo.....	1:100	1:4	7.2
	56	Frozen H ₂ SO ₄ in vacuo.....	1:200	1:8	7.2
	73	Air.....	1:100	0	7.3
	111	H ₂ SO ₄ in vacuo.....	4	6.6

NORMAL GOAT SERUM. BACTERIAL AGGLUTININ INDEX 1:200; CELL AGGLUTININ INDEX 1:12; pH 7.6

	Number Days Dried	Method of Drying	Bacterial Agglutinin Index	Hemag- glutinin Index	pH
Normal goat serum dried	17	Frozen H ₂ SO ₄ in vacuo.....	1:100	1:8	7.5
	65	Frozen H ₂ SO ₄ in vacuo.....	1:50	1:4	6.6
	65	H ₂ SO ₄ in vacuo.....

The serum of two guinea-pigs was treated in the same manner as serum from the horse and goat and the complement content tested. One lot of serum with a complement content of 0.02 c.c. to the dose failed to act after 15 days of drying when two and a half times this amount was used. The second lot with an initial strength of 0.03 c.c. failed to act in the higher doses after 11 days. These results are contrary to those of Shakell and Harris with regard to complement.

In combination with an emulsion of *B. dysenteriae*, complement was completely fixed by 0.15 c.c. of the original normal horse serum. After 16 days drying it required five times this amount of the frozen serum to bind the complement. Six times this amount of serum dried over sulphuric acid in vacuo without freezing acted very feebly on the 20th day.

In some instances, the H-ion concentration was determined by the colorimetric method. The results are given in the following table together with the agglutinating index of the corresponding specimen of serum. The dried serum was dissolved in 0.85% salt solution and diluted ten times with distilled water. The distilled water and the salt solution were tested and were neutral.

According to these data the H-ion concentration of the dried serum undergoes a slight change which does not become marked until the agglutinins have reached a very low point.

Burrows found, as quoted, that by his method a decrease of P_H occurred in the dried serum over the original serum. He makes no statement, however, regarding the time allowed to elapse before the reactions were taken, but pre-

⁸ Jour. Biol. Chem., 1916, 35, 431.

sumably they were taken on the completion of drying which should occur under 36 hours, while the above tests were after 17 days of drying.

Various phenomena that developed during the progress of the work indicate the possibility of many factors playing a part in the deterioration of immune bodies in the serum of the normal horse and goat, acting alone or interdependently.

The delay in the loss of agglutinins in the dialyzed serum points to salt concentration as one cause for this, either by effecting changes in surface tension of colloid particles or development of salt antagonism through interference with the law of direct proportions or variations in the electrolyte conditions. Application of the work of Osterhout⁹ and of Loeb¹⁰ on the subject of salt antagonism offers suggestions for further study of this phase of the problem. The delay of loss in frozen specimens as against the nonfrozen leads one to consider carefully the possibility of ferments being liberated and acting under the changed conditions. Another point that merits consideration is the relation of pseudoglobulins and euglobulins. The conversion of pseudoglobulins into euglobulins which plays such an important part in the concentration of antitoxin may find application to this problem of drying. Dean¹¹ thinks it quite possible that the pseudo- and euglobulins are different phases of physical aggregation in which case the many factors that may be acting during drying, especially salt concentration, might readily be the preceding cause.

SUMMARY

The normal antibodies in the serum of the horse and goat are gradually decreased by drying.

The agglutinins and hemagglutinins are less affected by drying if the serum is frozen while desiccation is going on.

The loss of titer in the nondialyzed serum takes place within the first 10 days while it is delayed in the dialyzed serum until after the 20th day.

The decrease of agglutinins in the dialyzed serum is uniform for all methods of drying until after 2 months, while in the nondialyzed serum the loss is greater in the serum that was not frozen and still greater in the serum dried in the air.

The P_H of dried serum is slightly greater than in the original serum and as the loss of agglutinins become greater the difference is more marked.

Dried serum dissolved and dried again shows definite changes in the formation of crystals.

It is probable that several factors rather than one alone are responsible for the changes in the antibodies produced by drying, these being of a physicochemical nature and acting interdependently.

⁹ Jour. Biol. Chem., 1918, 363; Science, 1916, n. s. 44, 318.

¹⁰ Jour. Biol. Chem., 1918, 34, 395; Science, 1912, 35, 112.

¹¹ Brit. Med. Jour., 1916, 2, 749.

THE PATHOGENICITY OF BACILLUS INFLUENZAE FOR LABORATORY ANIMALS

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INTRODUCTION

The severity and extent of the recent pandemic of influenza has stimulated many investigations of the etiology of this disease.

During the interval between the pandemic which started in 1889 and the one which began in 1918, the bacillus discovered by Pfeiffer¹ in 1892 was accepted rather generally as the cause of influenza. The etiologic relationship was questioned by some because the bacillus was frequently found in the normal throat and in connection with a number of other inflammatory conditions of the respiratory system. The finding of it under such conditions was assumed by some to be due to a diminution or loss, for the time being, of its virulence.

When influenza first appeared in extensive epidemic form in Europe in 1918, many bacteriologists were able to isolate the Pfeiffer bacillus from only a rather small proportion of the cases.² The same has also rather generally been true in this country. It is, therefore, the opinion of many that this organism is not the cause of the epidemic disease influenza.

On the other hand, the finding of this bacillus as the only organism in the lungs of many cases of influenza,³ as well as in the spinal fluid of a number of cases of meningitis, indicates that it has pathogenic properties.

Our knowledge as to how the Pfeiffer bacillus causes disease is as yet rather meager. The data obtained by experiments on lower animals have been rather conflicting. It was with the idea of determining more definitely what effect this bacillus has on laboratory animals and how such action is produced, that our investigation was undertaken.

EXPERIMENTS

Source of Cultures.—We worked with two cultures. One represented the only organism found in the cerebrospinal fluid of a case of meningitis which terminated fatally. The other represented a mixture of four different cultures

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¹ Deutsch. med. Wchnschr., 1892, 18, p. 28.

² Jour. Am. Med. Assn., 1918, 71, p. 1573.

³ Ibid., p. 1031.

kindly supplied to us by Dr. F. O. Tonney of the Chicago Health Dept. These were isolated from sputum during the recent epidemic of influenza. The organisms used were small nonmotile, gram-negative, nonspore-bearing aerobic bacilli which grew only on mediums that contained hemoglobin. The bacilli of young cultures had rounded ends and were well stained by fuchsin, but only slightly by methylene blue. Many showed bipolar staining. The colonies on oleate-hemoglobin agar were minute and discrete. The material used consisted of:

(a) Growths on oleate-hemoglobin agar⁴ after incubation at 37 C. for 24 hours suspended in normal salt solution. This medium, which yields a more profuse growth than ordinary blood agar does, is prepared as follows:

1. Prepare meat infusion agar (2%) having a reaction of 0.3-0.5% acid to phenolphthalein. Sterilize, 94 parts. (Liquefy before adding the ingredient named below.)

2. Add a 2% solution of sodium oleate which has been made up with distilled water and sterilized in the autoclave, 5 parts.

3. Add to the foregoing while still hot, a suspension of red blood corpuscles prepared as follows: Centrifuge sterile defibrinated human or rabbit blood; pipet off the supernatant serum; make up to original volume by the addition of sterile broth, 1 part.

4. Place in slanting position until solidified.

(b) Growths in oleate-hemoglobin broth, after incubation at 37 C. for 24 hours. This medium is prepared in the same way as the corresponding agar preparation except that meat infusion broth is substituted for the agar.

(c) Toxin of *B. influenzae* produced in oleate-hemoglobin broth and obtained by the following method:

1. Oleate-hemoglobin broth is inoculated heavily with a 24-hour culture of *B. influenzae* grown on oleate-hemoglobin agar.

2. Incubate for 24 hours at 37 C.

3. Centrifugate to throw down coarse particles.

4. Filter through a Mandler candle filter.

NOTE.—Use as soon after filtration as possible, since the toxin rapidly deteriorates.

(d) Toxin of *B. influenzae* produced in veal infusion blood broth and obtained by the following method suggested by Parker:⁵

1. Prepare a veal infusion broth neutral to phenolphthalein.

2. To 50 c.c. of this broth in an Erlenmeyer flask, add 5 c.c. of sterile defibrinated rabbit's blood.

3. Heat the mixture over a water bath at 75 C. until the blood coagulates and settles on standing. (This requires from 3-5 minutes after this temperature has been reached.)

4. Inoculate each flask with one or two slants of *B. influenzae* grown on oleate-hemoglobin agar.

5. Incubate for 24 hours at 37 C.

6. Centrifugate to remove coarse particles.

7. Filter through Mandler candle filter.

⁴ Ibid., p. 2050.

⁵ Ibid., 1919, 72, p. 476.

The animals used were rabbits, guinea-pigs and white mice. Animals varying in age and size were chosen. Inoculations were subcutaneous, intraperitoneal and into the blood.

Results of Inoculations.—The more significant data in connection with the inoculations and the results obtained are included in the tables.

The inoculations were followed, as a rule, within an hour—sometimes within half an hour—at other times, several hours, by indisposition. The symptoms were listlessness; loss of appetite; ruffling of the fur; rapid breathing; dyspnea; fever, and frequently also, twitching of muscles; convulsions; tonic spasms, resulting in retraction of the head; muscular weakness and loss of weight resulting, at times, in marked emaciation.

If the animal continued to live, the more acute symptoms usually passed off after two or three days. On the other hand, in spite of a reasonably good appetite, loss of weight and marked muscular weakness often continued for several weeks, after which some of the animals recovered, while others succumbed. Shortly before death the temperature would usually go below normal. Death, whether early or late, was usually preceded by convulsions. Fatal results occurred in from 1½ hours to 3 weeks. When the injections were made intraperitoneally, a peritonitis with marked tenderness of the abdomen frequently resulted. Some of the more significant records follow:

1. RABBIT 13.—Weight 517 gm., received an intravenous injection of 2 cc of a 24-hour oleate-hemoglobin broth culture from the "meningitis" strain of *B. influenzae*. Forty minutes later, the rabbit looked sick, was limp and respirations were deep and labored. In another 30 minutes, the animal was very weak, the head was retracted and presented jerky spasmodic movements. At this time there were also marked convulsions—the whole body shaking with clonic spasms. Breathing was rapid. Twitching of muscles accompanied by occasional convulsions and retraction of the head continued and became progressively worse for another two hours, at the end of which the animal died—three hours after the injection.

This is an example of the acute effects of the Pfeiffer bacillus as seen in a number of the younger rabbits. The symptoms are the same as produced in cases injected with the "toxin" alone. The effects are evidently the results of intoxication rather than infection. The strain of *B. influenzae* recovered from sputum produced similar effects.

2. MOUSE 15.—Received an intraperitoneal injection of 2 cc of an oleate-hemoglobin broth culture of the *B. influenzae*, isolated from the case of meningitis. Death occurred in 10 hours. A small amount of fluid was found in the peritoneal cavity. The Pfeiffer bacillus was isolated from the peritoneal fluid and the heart blood. Another bacillus which grew on plain agar was also isolated from the heart blood.

This is an example of the acute effects of the influenza bacillus. The presence of the bacillus in the blood indicates its power to invade or pass through tissue. The other organism found in the blood belonged to the colon group and was presumably derived from the intestinal tract of the mouse. Its invasion of the tissue was probably due to the lowered resistance caused by the injection of the influenza bacillus.

3. GUINEA-PIG 10.—Received an intraperitoneal injection of 5 cc of the toxin of the "meningitis" strain of *B. influenzae* grown in veal infusion blood broth. The temperature of the animal just before inoculation was 101.8 F.; the next day it was 104.2 degrees. It remained at about this point for 3 days, during which time the animal ate but little. After this time the appetite improved and

the temperature went down to 102 degrees. By the end of the seventh day, the weight had dropped from 597 to 440 gm.—a loss of 157 gm. He then received an intracardial injection of 5 cc of an oleate-hemoglobin broth culture. An hour later he was quite sick, and death occurred about 10 hours after injection. No *B. influenzae* were found, but another organism which grew on plain agar was obtained from the heart blood.

This experiment shows the acute and subacute effect of the "toxin"—characterized by indisposition, fever, loss of appetite and weight. The animal was on the road to recovery when the second injection was given. This produced acute symptoms. The presence of the organism recovered can no doubt be explained on the same basis as that given in connection with Mouse 15.

The more significant data of the more important experiments have been arranged in three tables. Each table represents the experiments for a different kind of animal. The nature of the material injected has already been explained. The letter "C" indicates that the material used was a mixture of four cultures obtained from Chicago through Dr. Tonney. In all other instances, the organism used was the one isolated from a case of Pfeiffer bacillus meningitis.

TABLE 1
EFFECT OF PFEIFFER BACILLUS ON RABBITS

Number Used	Size	Material Injected	Amount	Method	Death in	Remarks
3	Small	Veal broth	8 cc	Intra-venous	No effect. Control
4	Medium to large	Oleate broth	3-15 cc	Intra-venous	No effect. Control
3	Small	Oleate agar culture	½ tube in 3 cc salt solution	Subcutaneous	3 hours (one)	All sick
4	Small	Oleate agar culture	1 tube in 5 cc salt solution	Intra-venous	7 hours 12 hours 30 hours	Pfeiffer bacillus obtained from heart blood Pfeiffer bacillus obtained from heart blood One recovered but became weak and lost 135 gm. in 19 days
4	Small	Oleate broth culture	1-3 cc	Intra-venous	1¾ hours 3 hours 12 hours	Pfeiffer bacillus obtained from heart blood Pfeiffer bacillus obtained from heart blood One recovered
7	Small to medium	Oleate broth culture	6-8 cc	Intra-venous	6 days 18 days 3 weeks	Four recovered Foul smelling organism isolated Had marked meningeal symptoms
1	Small	Oleate broth culture	5 cc	Intraperitoneal	Recovered
1	Adult	Toxin-veal broth	4 cc	Subcutaneous	1½ hours	Several convulsions and rapid breathing
3	Adult	Toxin-veal broth	5-8 cc	Intraperitoneal	Very sick but recovered
5	Adult	Toxin-oleate broth	4-8 cc	Intra-venous	1½ and 1¾ hours	Three recovered
3	Half	Toxin-oleate broth	6 cc	Intra-venous	5 hours 4 weeks	Marked convulsions One recovered

TABLE 2
EFFECT OF PFEIFFER BACILLUS ON GUINEA-PIGS

Number Used	Material Injected	Amount	Method	Death in	Remarks
1	Oleate broth	5 c c	Intracardial	No effect. Control
2	Oleate broth culture	5 c c	Intracardial	10 and 19 hours	Pfeiffer bacillus obtained from the heart blood of one
1	Oleate broth culture	5 c c	Intracardial	19 hours	Pfeiffer bacillus recovered from heart blood
1	Toxin-veal broth	2 c c	Intracardial	Recovered
2	Toxin-veal broth	5 c c	Intracardial	3 days	Lost 99 gm. in weight; one recovered
1	Oleate agar culture	$\frac{1}{2}$ tube in 1 c c salt solution	Subcutaneous	Lost 134 gm. in weight
2	Oleate agar culture	1 tube in 5 c c salt solution	Intracardial	12 hours each	Another organism was isolated from heart blood of each
2	Oleate agar culture	1 tube in 5 c c salt solution	Intraperitoneal	23 and 48 hours	Another organism was isolated from heart blood of each

TABLE 3
EFFECT OF PFEIFFER BACILLUS ON MICE

Number Used	Material Injected	Amount	Method	Death in	Remarks
4	Oleate broth	2 c c	Subcutaneous (2), Intraperitoneal (2)	No effect. Control
4	Oleate broth	2 c c	Subcutaneous	9 hours 24 hours 3 days	One recovered; another organism obtained from heart blood of two
3	Oleate broth	2 c c	Intraperitoneal	8 hours 9 hours 24 hours	Another organism obtained from heart blood of one
2	Toxin-veal broth	2 c c	Intraperitoneal Subcutaneous	24 hours 10 days	Another organism obtained from heart blood of each
3	Toxin-oleate broth	2 c c	Subcutaneous	8 days	Two recovered
3	Toxin-oleate broth	2 c c	Intraperitoneal	2 days	Two recovered
2	Oleate agar culture	1/10 tube in $\frac{1}{2}$ c c salt solution	Intraperitoneal	3 days each	
2	Oleate agar culture	$\frac{1}{2}$ tube in 2 c c salt solution	Subcutaneous	22 hours and 4 days	
1	Oleate agar culture	$\frac{1}{2}$ tube in 2 c c salt solution	Intraperitoneal	48 hours	
2	Oleate agar culture and another organism	$\frac{1}{2}$ tube in 2 c c salt solution	Intraperitoneal	12 and 48 hours	The "contaminating" organism recovered from peritoneal fluid
1	Oleate agar culture and another organism	$\frac{1}{2}$ tube in 2 c c salt solution	Subcutaneous	21 hours	The "contaminating" organism recovered from heart blood

DISCUSSION

Our and to a certain extent also other experiments have shown that rabbits, guinea-pigs and mice are all susceptible to the Pfeiffer influenza bacillus. Other experiments have shown that monkeys⁶ and, to a slight extent, dogs,⁷ are also susceptible.

Rabbits.—Pfeiffer⁸ demonstrated as early as 1893 that rabbits were susceptible to this organism. As symptoms he emphasized dyspnea and muscle weakness. Delius and Kolle⁷ and Cantani⁹ have also demonstrated the pathogenicity of *B. influenzae* to rabbits. They mention especially the emaciation as a manifestation of the chronic cases. Of 32 rabbits injected by us with a culture or the "toxin" of the influenza bacillus, 15, or 46.8%, succumbed in from 1½ hours to 3 weeks after injection. The remainder were ill for a variable length of time.

Guinea-Pigs.—Of 10 guinea-pigs injected, 7, or 70%, succumbed in from 10 hours to 3 days. All of the pigs showed some signs of indisposition. Guinea-pigs appear to be more uniformly susceptible to the influenza bacilli than rabbits, although the higher mortality may be explained in part by the relatively larger doses injected. None of the pigs succumbed as promptly as did some of the rabbits. The susceptibility of guinea-pigs has previously been shown by Delius and Kolle⁷ and also by Kikuchi.¹⁰

Mice.—Of 24 mice injected by us, all but 4, or 83.3%, succumbed. This is in rather marked contrast with the recent statement of Spooner, Scott and Heath,¹¹ to the effect that "more than a hundred intraperitoneal injections of mice have shown that the organism is not pathogenic to that animal." On the other hand, Jacobsohn¹² succeeded in recovering influenza bacilli from the blood of mice provided they at the same time received an injection of dead streptococci or pneumococci.

The difference in the results obtained by different investigators is evidently due to a difference in the virulence of the organism and possibly also to the size of the dose.

Monkeys.—Pfeiffer and Beck⁶ succeeded in inducing coryza in a monkey by rubbing a pure culture on the unbroken mucous membrane

⁶ Deutsch. med. Wchnschr., 1892, 18, p. 465.

⁷ Ztschr. f. Hyg. u. Infektionskrankh., 1897, 24, p. 327.

⁸ Ibid., 1893, 13, p. 357.

⁹ Ibid., 1896, 23, p. 265.

¹⁰ Nippon-Eiseigakkwai-Zasshi, 1909, 3.

¹¹ Jour. Am. Med. Assn., 1919, 72, p. 155.

¹² Arch. de méd. expér., 1901, 13, p. 425.

of the nose. The symptoms were suggestive, but not at all typical of influenza in man. The introduction into the lungs of a suspension of the micro-organism into one monkey resulted in the development of fever and an illness which lasted for several days.

Wollstein¹³ succeeded in producing a meningitis in monkeys by the intraspinal injection of pure cultures of virulent strains of *B. influenzae*.

Dogs.—Delius and Kolle⁷ found that the subcutaneous or intraperitoneal injection of 50-60 agar cultures had no effect on dogs and that intravenous injections produced but slight symptoms. Larger doses, however, as also intracerebral injections of smaller ones, produced symptoms of intoxication.

We found that young (half-grown) rabbits and guinea-pigs were more susceptible than older ones. Delius and Kolle⁷ also found that small guinea-pigs could be infected and killed by cultures of the organism to which larger pigs were resistant. This fact, together with the difference in virulence and dosage, may account for the fact that Pfeiffer⁸ and more recently, Spooner, Scott and Heath¹¹ were unable to demonstrate any pathogenicity of this organism to guinea-pigs.

The route by which cultures or the "toxins" of the Pfeiffer bacillus were introduced into the body did not make as much difference as was to be expected. We found that in mice, the subcutaneous method produced results almost as quickly as the intraperitoneal method. In rabbits likewise there was no noticeable difference between subcutaneous, intraperitoneal and intravenous injections. Delius and Kolle⁷ found that guinea-pigs succumbed to $\frac{1}{4}$ of an agar culture when given intraperitoneally although it required the entire growth to produce a fatal result when introduced subcutaneously. The few intratracheal insufflations which we made were negative. Positive results in rabbits have, however, been obtained in this manner by Pfeiffer⁸ and by Spooner, Scott and Heath.¹¹

Pfeiffer⁸ succeeded in producing an inflammation by simply rubbing pure cultures on the unbroken nasal mucous membrane of a monkey and a rabbit. Delius and Kolle⁷ found that dogs were most susceptible when the bacteria were introduced intracerebrally, but slightly affected by intravenous, and still less so by subcutaneous and intraperitoneal injections.

¹³ Jour. Exper. Med., 1911, 14, p. 73.

We found that 2 c.c. of a broth culture or $\frac{1}{2}$ of an agar culture suspended in 2 cc of salt solution was usually fatal to mice. This is a large dose for such a small animal. Controls consisting of an equal amount of the sterile fluid medium did not produce any harmful effects. Five cc of a broth culture was usually fatal to both guinea-pigs and rabbits. The controls were invariably negative even when as much as 8 and 15 cc of the sterile broth were introduced into the ear vein of rabbits. In some instances 1, 2 and 3 cc doses of the broth cultures and $\frac{1}{2}$ of an agar culture were fatal to rabbits.

Various experimenters have found a marked difference in the virulence of influenza bacilli obtained from different sources. Some appear to be avirulent. Wollstein¹³ found that cultures of this organism from the meninges are distinctly more virulent to rabbits than are those from sputum. Our experiments tend to confirm this.

Kikuchi¹⁰ reports that he was able to increase the virulence of this bacillus by successive passage of the organism from one guinea-pig to another, using the peritoneal fluid of the succumbed animal for making the inoculations. The virulence was increased to the extent that whereas at first it required 8 agar cultures to produce the death of a young guinea-pig, after 6 successive inoculations, an injection of only $\frac{1}{10}$ of a cc of the peritoneal exudate was sufficient to kill the animal.

The injection of influenza bacilli or their "toxins" often resulted in the invasion of the tissues by another organism. This was no doubt due to the lowering of vitality produced by the influenza bacillus. We recovered two other organisms in this way. Injections of a mixture of the influenza bacillus and the other organism were more rapidly fatal than injections of the influenza bacillus alone.

Jacobsohn¹² was able to produce an influenza bacillus bacteriemia in mice only when using an impure culture of the organism.

Pfeiffer⁸ and also Delius and Kolle⁷ believed that the bacteria introduced intravenously were rapidly destroyed. Although we recovered influenza bacilli only from animals that succumbed within 30 hours after injection, we believe that the continuation of symptoms, especially loss of weight, weakness and convulsions can be explained on the basis that living organisms are present and continue to produce toxins. It is, of course, quite possible that the more chronic manifestations observed were due to the action of secondary invaders.

Pfeiffer⁸ found no evidence that the bacteria increased in number in the body. Delius and Kolle⁷ did not find any increase when the bacteria were injected intravenously into rabbits, but did find that they increased in number when introduced intraperitoneally into rabbits, guinea-pigs and mice, and intracerebrally in dogs.

We recovered the Pfeiffer bacillus from the heart blood after intraperitoneal injections—three times—once from each kind of animal used in the experiments. Pfeiffer⁸ was unable to produce a bacteriemia in rabbits when the bacteria were not introduced into the veins.

Cantani⁹ was unable to demonstrate invasion of tissue following subdural inoculations. On the other hand, Jacobsohn¹² succeeded in producing an influenza bacillus bacteriemia in mice if at the same time these animals received an injection of killed streptococci or pneumococci. Neither he nor Saathoff¹⁴ were able to isolate the influenza bacilli from the blood of mice when only pure cultures were used. Evidently the invasive power of the influenza bacillus depends, not only on the virulence of the organism as in our case, but also on the lowering of the resistance of the body by other bacteria.

The production of pronounced symptoms within a few hours after inoculation must be due to a marked, often profound, intoxication. Pfeiffer⁸ and other earlier investigators believed that this was due to an endotoxin liberated as the result of the rapid destruction of the bacilli in the body. Pfeiffer noticed no difference in the results when rabbits were inoculated with living bacteria or with cultures killed by chloroform. He believed therefore that it was not a case of infection but rather of intoxication.

Delius and Kolle⁷ came to practically the same conclusion. They also found that the filtrate of killed cultures was toxic, although through instability, its toxicity was rapidly lost. Cantani⁹ demonstrated that cultures killed by heating to 60 C. were fatal to guinea-pigs when injected either intracerebrally or intraperitoneally.

Slatineau¹⁵ obtained what he regarded as the endotoxin by the following method: A suspension of an agar culture in salt solution was centrifugated. The sediment was treated with equal parts of fresh horse serum and distilled water. This was left in the incubator for 12 hours and again centrifugated. The supernatant fluid, supposed to contain the endotoxin liberated through the destruction of

¹⁴ München. med. Wehnschr., 1901, 54, p. 2220.

¹⁵ Centralbl. f. Bakteriol., I, O., 1906, 41, p. 185.

the bacteria by the serum water, was found to be toxic to guinea-pigs. He also found that the bacteria themselves after the treatment referred to were still toxic.

Parker⁵ succeeded in obtaining a poison produced by the influenza bacillus by filtering veal infusion blood broth cultures after a period of incubation of from 6-20 hours. Two c.c. of the poison killed a medium sized rabbit in from 1-3 hours. The poison deteriorated very rapidly even when kept in an icebox. She was also able to make rabbits immune to this poison. The immune serum appeared to be antitoxic in nature.

We have been able to confirm Parker's experiments with reference to the production of a filtrable poison. An equally effective poison was produced in oleate-hemoglobin broth. We believe the production of the poison to be too rapid to be explained on the basis of an endotoxin alone. Death of the animal was produced, as a rule, more promptly when using broth cultures than when using an equal volume of the filtrates. From the fact that agar cultures suspended in salt solution were almost as effective in producing symptoms and death as a broth culture of corresponding size, we conclude that the disease is more of an infection than was held to be the case by previous investigators and that toxins, chiefly extracellular, are produced in the body of the inoculated animal.

SUMMARY AND CONCLUSIONS

The Pfeiffer influenza bacillus is distinctly pathogenic to mice, guinea-pigs and rabbits. This quality is apparently limited to certain cultures or strains of this micro-organism.

The guinea-pig is more uniformly susceptible to the influenza bacillus than rabbits and mice, although not as susceptible as some rabbits. Rabbits show a greater variation in individual susceptibility.

Young (half-grown) animals are more susceptible than larger ones.

Intravenous and intraperitoneal injections are slightly more rapidly fatal than subcutaneous injections.

Two c.c. of a 24-hour hemoglobin broth culture of a virulent organism is fatal to about 90% of white mice. Five c.c. of such a culture is fatal to about 50% of rabbits and 70% of guinea-pigs.

Death of animals occurs in from 1½ hours to 30 days after injection. It is probable that deaths occurring after the fourth or fifth days may be caused by secondary invading organisms rather than by the Pfeiffer bacillus.

The chief acute symptoms are listlessness, muscular weakness, rapid and labored breathing, elevation of temperature and convulsions. The chief chronic symptoms are loss of weight and muscular weakness.

The virulence of the organism varies with cultures from different sources.

The injection of influenza bacilli favors the invasion of tissue by other bacteria. Likewise the introduction of other bacteria favors the proliferation in the body and the invasion of tissues by the influenza bacillus.

Influenza bacilli are, as a rule, apparently rapidly destroyed soon after introduction into the body. Following injection into the peritoneal cavity they may appear in the blood. This appears to be dependent on either the virulence of the micro-organism or a condition of lowered resistance on the part of the body.

The influenza bacillus produces a toxin which is fatal to mice, guinea-pigs and rabbits almost as rapidly as are broth cultures of equal dosage. This toxin is produced very rapidly and can be obtained by filtering broth cultures. It is not possible to state definitely whether it is an endotoxin or an extracellular one.

Although the symptoms of intoxication as seen in lower animals following injections of the Pfeiffer bacillus are suggestive of the profound intoxication seen in connection with many cases of the epidemic disease influenza in the human being, these experiments do not furnish any proof that the Pfeiffer bacillus has any specific etiologic relationship to that disease. On the other hand, they suggest that a possible etiologic relationship cannot be ignored.

ADJUSTMENT OF REACTION OF CULTURE MEDIUMS

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For the titration of culture mediums, the use of phenolphthalein as an indicator, determining the total acidity, is rapidly becoming obsolete. This does not apply, without qualification, to the mediums for the growth of *B. typhosus*, *B. paratyphosus*, staphylococcus and such other organisms that have a wide range of selectivity in cultural requirements; it does, however, apply to the pneumococcus, streptococcus, *B. influenzae*, meningococcus and the more strictly parasitic organisms that have a comparatively narrow range of selectivity in cultural requirements.

These facts were forcibly brought to our attention when it developed on us to produce large quantities of pneumococci in a serum-free broth. It has been the custom to grow pneumococci in infusion broth that has been titrated to + 0.2 (0.2% acid) to phenolphthalein, the titration being done at the boiling point, and a very faint shade of pink being considered the neutrality point. It is very difficult for even the experienced worker to determine accurately this neutrality point, and in a highly colored broth, the difficulty is even greater.

Hiss and Zinsser say that "the most favorable reaction of media for the cultivation of this organism (pneumococcus) is neutrality, or moderate alkalinity (two-tenths to eight-tenths per cent. alkalinity to phenolphthalein). Slight acidity, however, if not exceeding eight-tenths per cent., does not materially hamper development."

Beef infusion broths were therefore made accordingly, but while some batches of broth gave good results, others partially or wholly failed to grow the pneumococcus. Batches of greater alkalinity were tried—these gave very poor results; batches of greater acidity were tried, and while the results were better, great irregularity of growth was encountered.

The method of titrating the broth by the hydrogen-ion concentration method was then tried, and found to be wholly practical and simple. Once the proper degree of alkalinity, measured in terms of hydrogen-ion concentration (which is more simply expressed by the

symbol P_H) was determined, profuse and consistent growth of the pneumococcus was secured. When this optimum reaction for the pneumococcus had been found to be P_H 7.8 to 8.0, this broth was found to be + 3.0 to + 3.5 (3-3.5% acid) to phenolphthalein, a total acidity far beyond anything previously suggested for the growth of pneumococcus. Our experience with the meningococcus was similar to our experience with the pneumococcus. A glucose, beef infusion agar, titrated to neutrality with phenolphthalein, as well as slight degrees to either side of the neutrality point, failed to give us luxuriant growth with any degree of consistency. It was only when such an agar had been set in reaction by the P_H method that profuse growth could be obtained with absolute regularity.

Much has been written of hydrogen-ion concentration during the past few years.¹ This has been, in the main, couched in highly technical terms of chemistry and mathematics. Shorn of this impressive verbiage, this system of titration resolves itself into a simple, highly practical and accurate method. It is true that a complete understanding of the underlying principles demands close study and application; it is further true that if one were interested in all the reactions of substance, ranging from a normal HCl on one side to (P_H 0.0) to a normal NaOH (P_H 14.0) on the other, it would necessitate the possession of a large number of solutions or reagents. It is still further true that the bacteriologist is interested only in the neutrality point and in those reactions lying just to either side. Bearing in mind that a P_H of 7 is absolute neutrality, and that 7.3-7.5 is the approximate reaction of normal blood, it is seen that a range of reactions from 6.6-8.4 is all that is necessary, for practical purposes, in bacteriology.

To set the reaction of a broth is a simple matter. A measured 10 c.c. of the broth are taken in a test tube that has been rinsed with a portion of this broth. One-half c.c. of a 0.02% solution of phenolsulphonephthalein—frequently referred to as phenol red—is added, and from a burette a measured quantity of N/20 NaOH is added until the color matches the standard chosen. Simple calculation then determines the amount of N/1 to be added to the entire quantity of broth to bring it to the reaction of the chosen standard. This standard—say 7.8—consists of a solution of KH_2PO_4 and NaOH in distilled water with phenol red. This water is double distilled and the combination of pure salts is very accurately weighed out. It is the preparation of these standards (a graded solution of these salts with color indicator for each p_H desired, about 8-10 being necessary for bacteriologic work) that has heretofore presented the greatest difficulty.

Under ideal conditions these standard solutions would be made up daily, with specially distilled water, with salts of greatest purity, very accurately weighed out, and the complete salt solutions, or standard, tested for P_H accuracy by an electrometric method.

If a chemist is available, it is possible to have him prepare the 10 different salt solutions, ranging from 6.8-8.4, each in a quantity of 200 c.c., and these

¹ Tizard: Brit. Assn. Report, 1911, p. 268; Washburn: Principles of Physical Chemistry, p. 333; Clark and Lubs: Bacteriol., 1917, 2, p. 1. Sørensen: Biochem. Zeitschr. 1909, 21, p. 131. Findlay: Practical Physical Chemistry, p. 187 et seq.

solutions, with proper care, could be used over an extended period of time. The weights and measures for these standards are appended.

To make color standards of these solutions it is necessary only to measure 10 cc of each solution into a chemically clean test tube, add 0.5 cc of the 0.02% solution of phenol red, and cork the tube. The material under titration is brought to the same color shade as the color standard that represents the desired P_H .

The fact that commercial laboratories² are now preparing, at a reasonable price, these standard color solutions, sealed in ampules, and sufficient phenol-sulphonephthalein for extended use, makes the entire process so simple, and so much more accurate than the old-fashioned phenolphthalein titration, that there is no longer an excuse for any laboratory, no matter how small or isolated, to neglect this method.

The ampuled standards present a graded series of shades of yellow, pink and red; deep red appears on the alkaline or 8.4 side, and pale yellow to faint pink on the acid or 6.6 side. These standards may be used with safety for about 6 months, during which time, when not in use, they are to be kept in the dark at an even temperature.

Such a series of ampules, along with a combined rack and comparator are prepared at the Army Medical School and issued for use in the service.

When a medium of a given P_H is desired, the standard color ampule of the desired P_H is selected and set aside. Into a tube of the same diameter a measured 10 cc of the ampule, rinsed out with the medium to be titrated, are placed and to this are added 0.5 cc of the solution of phenol red. From a burette a solution of NaOH, known to be 1/20 strength of a concentrated solution, is added until the color deepens and finally matches the standard selected.

In order that the native color of the medium under titration be discounted, a simple comparator is used. It is merely a block of wood with 4 holes to receive 4 test tubes, and with 2 horizontal holes, each piercing 2 of those that receive the tubes. A dipping in black paint improves it. In the 2 front holes are set the tubes of the medium to be titrated. To the tube on the left, which contains 10 cc of this medium are added 0.5 cc of the phenol red solution and the contents mixed by a swirling motion. Behind this tube on the left is set a tube of distilled water; behind the tube on the right is set the standard to which it is desired to adjust the broth. In this fashion, a direct comparison may be made by glancing through the horizontal holes, the eye seeing on each side the same total contents; i. e., distilled water, color indicator, medium and glass. Dilute NaOH is now added from a burette, to the front tube on the left, until the colors match. Simple calculation determines the amount of concentrated NaOH, 20 times the strength of the dilute, necessary to bring the bulk of the medium to the proper adjustment. After the addition of this concentrated NaOH, it is best, after thoroughly stirring, to test a sample of the adjusted medium, by placing 10 cc in a tube, adding 0.5 cc of the indicator, phenol red, and placing in the comparator. Any deviation may be corrected by the addition of more NaOH or HCl, until the color matches absolutely.

It is convenient to use, for the stock 0.02% solution of phenol red, a bottle fitted with a perforated stopper, through which has been thrust a 1 cc pipet. This pipet is to be covered with a bit of tinfoil when not in use.

Daylight, direct or indirect, is to be used for comparisons; if artificial light is necessary, a nitrogen bulb and ground-glass screen should be used.

² Hynson, Westcott & Dunning, Baltimore, Md., supply such standards and indicators.

When one has become accustomed to working with these color standards one can almost memorize the shades of color representing certain P_H 's much in use. If one should be wholly without standards, and have only a 0.02% solution of phenolsulphonephthalein (renal test ampules, containing 6 mg. are usually available), recourse may be had to a very valuable method brought out by Barnett and Chapman.³ A tube, containing 10 cc of a very weak acid (about N/40 HCl) and another containing 10 cc of a very weak alkali (about N/20 NaOH) have added to each 0.25 cc of the indicator solution, phenol red. The acid tube becomes quite yellow, the alkaline tube becomes quite red. Looking through both of these tubes simultaneously, a shade of pink representing a P_H of 7.8 or 7.9 is seen.

Barnett and Chapman have carried their idea further and have devised a method whereby it is possible to obtain approximate accuracy with the other degrees of hydrogen-ion concentration within the phenol red range.

Six tubes each containing 10 cc of the weak acid have added to them, in order, the following quantities of the phenol red solution: 0.45, 0.4, 0.35, 0.30, 0.25, 0.20 cc; six tubes of the weak alkali have added to them, in order, the complementary quantities, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 cc. Looking through the first pair of tubes (not mixing them) they state that the p_H represented is 6.9 while the last pair of tubes represents 8.1. The entire range is 6.9, 7.2, 7.5, 7.7, 7.9, 8.1. Kligler⁴ has determined the result to be 7.1, 7.3, 7.5, 7.7, 7.8, 7.9. It has been our experience that such combinations yield the following figures: 7.0, 7.3, 7.5, 7.7, 7.8, 8.0. The slight disparity in the results shows that while slight variations do occur and make the method not absolutely accurate, the error is so slight as to be of little importance in the adjustment of culture mediums.

The use of 2 tubes to make a color standard, would necessitate the use of a comparator, to receive 3 tubes instead of 2, a matter simple to rectify.

Liquid mediums, such as broth, may be titrated either hot or cold; cold is to be preferred, for it more closely simulates the conditions under which the bacteria will be grown.

Agar mediums are best adjusted in the broth stage, and the agar added as the last step. Agar is, for all practical purposes, neutral in reaction, and does not change the p_H of the broth. If this should not be convenient, the agar may be melted and titrated hot. In comparing the colors with agar it is best to wait until the agar has set before concluding that the colors are identical. It is a simple matter to send this tubed specimen, with indicator in it, along with the batch of mediums, through the sterilization process to check the reaction after sterilization.

Relatively large quantities of blood serum, or whole blood, added to an alkaline or acid medium, tend to approximate the final P_H to 7.3 or 7.5. Besides this, because of the balanced basic and acid salts contained in blood serum, such an addition of serum tends to hold stable the hydrogen-ion concentration of a medium, during bacterial growth. These salts are usually referred to as "buffer salts." The addition of blood serum may be imitated by the addition of about 0.5% K_2HPO_4 to a medium, thereby increasing its "buffer salt" content. An appreciable amount of acid must then be produced by the growing organisms before the medium becomes more acid. Over and above the advantage of the nutrient properties of the serum, this accounts in part for some of the evident superiority of blood mediums.

³ Jour. Am. Med. Assn., 1918. 70. p. 1062.

⁴ Jour. Bacteriol., 1919, 4, p. 35

The usual methods of sterilizing medium—the Arnold and the autoclave—serve to change the P_H of mediums but slightly. We have found simple nutrient (extract) agar to change but 0.2 to 0.4 of a P_H . With the infusion mediums, in which there is a higher proportion of the so-called buffer salts—the natural salts of the meat—the change is usually about 0.2, though very frequently there is no change at all.

There is no constant interrelationship between p_H readings and phenolphthalein titrations, except in a case where a definite relationship has been established, in a medium of absolutely standard construction, using distilled water, the same batch of meat extract and peptone. Any change in technic changes the relationship between the two systems of titration; as a matter of fact, the p_H is so satisfactory and practical, that this relationship is only of academic interest.

It now develops that most of the organisms of interest to the bacteriologist have well defined limits of p_H in which they will grow, as well as an optimum. This subject has received increasing attention during the past year, and it will not be long before the optimum and limits of all the important organisms will be well defined in terms of p_H .

B. typhosus and *B. paratyphosus*.—These have wide ranges of p_H in which they will grow, and therefore an ill defined optimum. These organisms will grow on an agar as alkaline as P_H 9.6, and on agar of an acidity of P_H 4 (it would be better to say *in* agar, for at P_H 4 agar will not solidify). The optimum ranges from 6.2-7.2.

Making such simple nutrient agar with Armour's extract and peptone, the final reaction, without adjustment, is usually P_H 6.3. This is really ideal for the growth of *B. typhosus*. Such a 3% agar, at P_H of 6.3 offers an excellent surface for inoculation; this same agar, at a P_H of 4 will not solidify. At 4 it is fluid, at 4.2 it is thick and slushy, at 4.4 it is very soft, and much water of syneresis collects, 4.6, 4.8 and 5 present agars of soft consistency and crumbling surface. From 5.2 on, the agar becomes "workable." As one goes from 6.3 toward 4 the agar becomes softer and finally useless; as one goes from 6.3 toward 9.6 the agar becomes more and more cloudy, from precipitated phosphates, until at about 8 it becomes very unsatisfactory. Therefore a plain nutrient agar, or trypsin agar, of from 6.2-7.2 offers the best reaction for this medium measured by both the characteristics of the medium and its nutrient qualities.

What has been said for *B. typhosus* holds good for *B. paratyphosus* A and B, except that these organisms thrive better in the more alkaline reactions than does the typhoid bacillus. *B. paratyphosus* B, in addition, thrives much better in very acid mediums (4) than do the other two.

It might be of interest to note that the faculties of *B. paratyphosus* A and B, for fermenting minimal quantities of sugar, with gas production, seem also to have P_H limits (for *B. paratyphosus* A from 5.4-7.8, and for *B. paratyphosus* B from 4.8-8.4), with the optimum, for these two organisms, with regard to gas formation, fairly well defined. For *B. paratyphosus* A this optimum is from 6.3-7.4; for *B. paratyphosus* B this optimum is from 5.2-7.6.

It is well to bear these facts in mind for the construction of Russell's double sugar. With this medium, on the lower side one is limited by a soft agar and a red medium, on the upper side one is limited by a too blue

medium. Kligler⁶ has recommended P_H 7.4 for Russell's double sugar medium. We have found either 7.4 or 7.6 to be very satisfactory, since this point gives optimum growth for typhoid, B. paratyphosus A and B, paratyphosus B, colon and dysentery and is well within the optimum gas range of the two latter organisms. At this P_H , also, litmus has the delicate lavender tint necessary for this medium. Kligler recommends P_H 7.8-8.0 for the construction of Endo mediums.

Norton⁶ has called attention to this subject of hydrogen-ion concentration adjustment estimation of culture mediums, and has collected some of the data that have appeared during the past year. Kligler recommends a p_H of 7.0-7.2 for brilliant green agar. Meyer and Stickel⁷ recommend a slightly more acid reaction for brilliant green mediums, 6.4-7, and for their peptic digest mediums, for typhoid bacillus, 7.0-7.2. The latter is within the range of phenol red, i. e., 6.6-8.4.

The members of the committee of the American Public Health Association⁸ in their standard method of examining disinfectants, recommend P_H 6.0-7.0, optimum at p_H 6.5.

The Pneumococcus.—At the time we found it necessary to determine the optimum P_H for the pneumococcus, the figures of Dernby and Avery⁹ were not available. We found that the following broth gave excellent and consistent growth. One pound of ground beef in 1 liter of water is heated to 55 degrees for one hour, after which it is filtered through cotton. Armour's peptone, 1%, and NaCl, 0.5%, are then added, and the broth is brought to boiling to dissolve the peptone. The broth is then set to P_H 7.3-8.0, after which it is filtered through paper. In liter quantities it is autoclaved 1 hour, and the reaction changes hardly at all. Records were kept on several hundred batches of broth, made up in this standard fashion. While the figures fluctuated slightly, the average run of broth presents the following data:

Native phenolphthalein reaction of broth = + 4.3%; native p_H = 6.4.

Corresponding total acidity = + 2.4%; set to = 7.8

Autoclaved, 1½ liter lots, 15 lbs., 60 minutes

Total acidity immediately after autoclaving = + 2.7%; p_H = 7.5.

Total acidity after 24 hours' incubation at 37 C. = 3.5%; p_H = 7.8.

This broth gives rich and consistent growth of the pneumococcus and the addition of 0.5% glucose makes 5-7 billion per cc possible. We found that 8.2 was the alkaline limit, while 7.2 was about the acid limit. These facts are closely in accord with Dernby and Avery. While it is difficult to plant a glucose broth of P_H 7.2 and consistently get growth, pneumococci, planted in a P_H 7.8 glucose broth will continue to grow until they have produced a P_H of about 5.2. This applies not only to types I, II and III, but also to type IV. There is reason to believe that this end point of acid production may be a differentiating factor in the streptococci; there is reason to hope that it may assist in differentiating pneumococci from streptococci.

Streptococci.—All the strains of *Streptococcus viridans* with which we have had to deal, have grown well in a P_H 7.8-7.6 infusion broth, though some strains remain suspended in an even emulsion, others sediment out, in growth.

⁶ Jour. Exper. Med., 1918, 28, p. 319.

⁶ Amer. Jour. Pub. Health, 1919, 9, p. 3.

⁷ Jour. Infect. Dis., 1918, 23, p. 53.

⁸ Amer. Jour. Pub. Health, 1918, 8, p. 506

⁹ Jour. Exper. Med., 1918, 28, p. 345.

Blood agar, for pneumo- and streptococcus, may be made by adding agar to the preceding broth, and, after sterilization, the requisite amount of corpuscles, and consistent growth may be expected.

Influenza Bacillus.—The cultivation of the *B. influenzae* seemed to have offered considerable difficulty, early in the epidemic, and may be accountable for some of the widely varying reports. The development of the so-called "chocolate agar," an infusion agar, to which laked blood is added while the agar is 90 C., removed many of the difficulties. If this infusion agar be set to p_H 7.8 or 8.0¹⁰ rather than the untrustworthy 0.2% acid to phenolphthalein, even more regularity in results may be expected.

B. Cholerae.—This organism grows well on the ordinary extract agar, or broth, within a range from 5.6-9.6+. Its optimum is about from 6.2-8.0.

B. Dysenteriae.—This organism grows well on plain agar, within a range from 5.4-9.6+. Its optimum is about from 6.3-7.8. There seems to be no different selectivity on the part of the Shiga, Flexner or Y strains, except that Shiga does not grow quite so well in the more acid mediums.

Meningococcus.—Great difficulty was found in growing the meningococcus on a serum-free infusion glucose agar, titrated by phenolphthalein, when this organism was desired in large quantities for vaccine production. When the P_H system of titration was substituted, the problem became comparatively simple. The meningococcus, on such a medium, has a rather narrow range of good growth—from 7.4-7.8, with the optimum rather definitely at 7.6. This is in agreement with Gates¹¹ who states that "a reaction favorable to the meningococcus cannot be determined from the total titrable acidity (phenolphthalein system) but depends solely upon the hydrogen-ion concentration of the medium." He states that in a serum dextrose broth, the range lies between 6.1 and 7.8, the optimum at 7.4.

M. Melitensis.—It does not produce luxuriant colonies except between P_H 's of 6.3 and 8.4, the optimum being between 6.6 and 8.0.

Gonococci.—Even those strains that have been a long time on artificial mediums, have rather well defined limits of growth, and rather sharply defined optimum. One of many batches, of Vedder's starch agar, that seemed to grow those gonococci exceptionally well, had a P_H of 7.5. Subsequently, 4 batches were made, soluble starch, prepared by Small's method¹² being used, and set to P_H 7.8, —8, —8.2, —8.4. These were autoclaved at 10 lbs. for 20 minutes, and yielded titrations of 7.6, —8, —8.2, —8.4. While the latter 3 batches produced growth, the 7.6 agar was far superior. Readings were made after 36 hours, the tubes melted, along with the growth, and p_H estimations were made. The reaction had not changed appreciably.

Indicators.—The number of color indicators to denote acid production by bacteria is now becoming legion. Two well-known popular ones are rightly so, as far as true record of acid produced is concerned. Litmus and Andrade, both frequently used, are excellent indicators of the true neutral point, and slight acid production.

Litmus at a P_H of 6.6-6.8 presents a reddish violet, at 7 it is a delicate lavender, and at 7.2-7.4 it is bluish violet.

Andrade at 7.4 is quite colorless, at 7.2 and 7 it is very faint pink, while at 6.8 and 6.6 the magenta is very definite.

¹⁰ Hitchins, A. Parker: Personal communication.

¹¹ Jour. Exper. Med., 1919, 29, p. 321.

¹² Jour. Am. Chem. Soc., 1919, 41, p. 1.

Peptones.—There is a wide variation in reaction among the peptones in common use; there is even a variation between batches of the same brand.

Of several samples titrated in a 1% solution, we find Witte 7, Armour's 6.6, Difco 7.3 and Fairchild's 4.9. These same samples, titrated cold, with phenolphthalein as indicator, yield the following: Witte 0.4% acid, Armour 0.7% acid, Difco 0.3% acid, and Fairchild 1.25% acid.

Sugars.—It might be of interest to know that 3 samples of lactose, in distilled water, sterilized by Arnold and autoclave, yield the following results: Sample 1 received 1 hour in Arnold on 3 consecutive days; sample 2 received in autoclave 15 lbs., 30 minutes; sample 3 was unheated. All three samples had a P_H of 4.2. On boiling the tubes vigorously to remove carbon dioxide, the 3rd sample returned to neutral, or P_H 7, the first 2 heated samples remained unchanged. Sterilization seems to produce some permanent change in reaction, though glucose was absent in these specimens.

TABLE 1
STANDARD SALT SOLUTIONS

pH 6.6	50 cc m/5 KH_2PO_4	17.80 cc m/5 NaOH	Dilute to 200 cc
6.8	50 cc m/5 KH_2PO_4	23.65 cc m/5 NaOH	Dilute to 200 cc
7.0	50 cc m/5 KH_2PO_4	29.63 cc m/5 NaOH	Dilute to 200 cc
7.2	50 cc m/5 KH_2PO_4	35.00 cc m/5 NaOH	Dilute to 200 cc
7.4	50 cc m/5 KH_2PO_4	39.50 cc m/5 NaOH	Dilute to 200 cc
7.6	50 cc m/5 KH_2PO_4	42.50 cc m/5 NaOH	Dilute to 200 cc
7.8	50 cc m/5 KH_2PO_4	45.20 cc m/5 NaOH	Dilute to 200 cc
8.0	50 cc m/5 KH_2PO_4	46.80 cc m/5 NaOH	Dilute to 200 cc
8.2	50 cc m/5 H_3BO_3	m/5 KCl	Dilute to 200 cc
8.4	50 cc m/5 H_3BO_3	m/5 KCl	Dilute to 200 cc

TABLE 2
REACTIONS OF CULTURE MEDIUMS FOR VARIOUS ORGANISMS

Medium and Organism	Acid Limit	Alkaline Limit	Optimum
Russell double sugar for <i>B. typhosus</i> , <i>B. paratyphosus</i> A and <i>B. dysenteriae</i> and col.	7.0	7.8	7.4-7.6
Endo's medium for intestinal flora	7.8-8.0
Brilliant green	6.4	7.2	6.8-7.0
Simple nutrient agar for typhoid	4.0	9.6+	6.2-7.2
<i>B. paratyphosus</i> A	4.0	9.6+	6.2-7.2
<i>B. paratyphosus</i> B	4.0	9.6+	6.2-7.2
Dysentery, Shiga	4.8	9.6+	6.2-8.4
Dysentery, Flexner	4.8	9.6+	6.2-8.4
Dysentery, "Y"	4.8	9.6+	6.2-8.4
Cholera vibrio	5.6	9.6+	6.2-9.0
<i>M. melitensis</i>	6.3	9.6	6.6-8.2
Infusion broth for pneumococcus	5.0	8.0	7.8
<i>Streptococcus hemolyticus</i>	4.5	8.0	7.6-7.8
<i>S. viridans</i>	4.5	8.0	7.6-7.8
Infusion glucose agar for meningococcus	7.4	7.8	7.6
Chocolate medium for <i>B. influenzae</i>	?	?	7.8
Vedder's starch medium for gonococcus	7.0	8.0	7.4-7.6

CONCLUSION

The adjustment of bacteriologic culture mediums according to hydrogen-ion concentration, because of its accuracy and simplicity, should wholly supplant the phenolphthalein (total acidity) method.

COMPLEMENT FIXATION WITH ACID-FAST BACTERIA

I. A STUDY OF VARIOUS ORGANISMS WITH IMMUNE RABBIT SERUMS

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Since the discovery of the fixation reaction by Bordet and Gengou in 1901, there has been a lively interest in the application of the test to the recognition of specific antibodies to acid-fast bacteria, particularly the organisms causing tuberculosis and leprosy. It was while studying fixation in tuberculosis that Wassermann and Bruck in 1905 devised their well-known method for the serodiagnosis of syphilis. Following this discovery, the attention of workers in serology was largely taken up by the study of the Wassermann reaction so that several years elapsed before complement fixation with acid-fast organisms again became the object of much interest. Within the past few years, however, many workers have been experimenting with various tubercle antigens until at the present time the fixation reaction in tuberculosis, in the hands of several workers at least, is apparently specific and of considerable value in diagnosis.

Obviously much of the importance of complement fixation with bacterial antigens depends on the specificity of the antigens. With acid-fast antigens there has been much confusion, especially in the interpretation of results, because the specificity of such reactions is obscured by a rather widespread "group reaction." It has not been possible to distinguish various members of this group from each other with immune serums except in a very inconclusive and unsatisfactory manner. This group reaction has been assumed to be due to some antigenic substance common to the various acid-fast bacteria. For example, the serum of cattle with chronic paratuberculous enteritis (Johne's disease) will react by the fixation test almost equally well with antigens of the causative acid-fast bacillus and with human, bovine and avian tubercle bacilli. The lack of a clear-cut specificity is further illustrated by the fact that much of the earlier work on complement fixation in tuberculosis was hampered by the discovery that

many cases of syphilis without evidence of tuberculous infection gave positive fixation with tubercle bacilli and tuberculin antigens. Many observers have noted also that a number of cases of leprosy give fixations with various lipoidal tissue extract antigens used in the Wassermann test.

That the difficulties in the correct interpretation of the results of fixation reactions with acid-fast antigens are not fully appreciated by some observers may be illustrated by reference to the acid-fast bacilli that have been described and grown by numerous investigators from lepers. The organisms isolated by different observers have not been identical, and, since none has fulfilled Koch's requirements, the artificial cultivation of the true Hansen bacillus is not generally accepted as proved. Several of the acid-fast organisms have been used as antigens in fixation tests, and on finding fixation with leper serum it has been assumed that this was a specific reaction. In view of the group reaction, the attempt to justify the claim of the identity of any acid-fast organism with the Hansen bacillus by such indirect evidence is very unconvincing.

The entire question of complement fixation with acid-fast antigens, therefore, although not well understood, is one of considerable importance, and it is only by careful studies of the serum of immune animals, as well as the serum of patients suffering from various infections, that the value of these reactions can be determined and their specificity properly estimated.

A review of the experimental work in which acid-fast bacteria have been used as antigens, emphasizes the difficulty in interpretation of the results of fixation with this group in so far as specificity is concerned. While a large number of studies have been made with single organisms, especially tubercle bacilli, the work in which several acid fast bacilli have been compared antigenically is confined to relatively few papers which will be reviewed in the following paragraphs.

Gengou¹ immunized guinea-pigs against acid-fast organisms: the butter bacillus of Rabinowitsch (2 strains), of horse dung, timothy hay, Korn I, Tobler II, Tobler V, fish tuberculosis, blindworm tuberculosis, Arloing's bacillus of homogeneous tuberculosis, human, bovine and avian tubercle bacilli. The organisms were grown on glycerol potato, dried over KOH, powdered and suspended in salt solution. Immunization was carried out by 3 subcutaneous injections of 5 mg. each and the animals bled from 14 to 21 days after the last injection. Not all possible cross fixations were done except with the

¹ Berl. klin. Wchnschr., 1906, 43, p. 1531.

serum of the animals immunized against human tuberculosis. The immune serums of human, avian and bovine tuberculosis showed fixation with almost all the antigens used, while the cross fixations with other serums and acid-fast antigens were positive with few exceptions. A normal guinea-pig serum gave fixation with the timothy hay antigen so that it may have been anti-complementary. He concludes that the serum of guinea-pigs injected with acid-fast bacilli form sensitizers, not only against homologous bacilli, but also against the other acid-fast bacilli, especially human, bovine, and avian tuberculosis. This rule presents only rare exceptions.²

Much and Hoessli³ used as antigens washed bacillary emulsions of organisms grown in broth, ground and suspended in phenolized salt solution. The strains studied were bacillus of human tuberculosis, urine bacilli (smegma?), blindworm bacilli and bacillus of timothy hay. Of serums from 100 cases of proved tuberculosis, 30 reacted positively with the tubercle bacillus. Of these, 19 were tested with the 3 other antigens and 13 reacted positively with urine bacilli, 4 with blindworm antigen, while none gave fixation with timothy hay bacillus. In addition, the serums of two rabbits immunized with urine bacilli gave fixation with urine bacilli, blindworm bacilli and human tubercle bacilli and also with tuberculin and a broth filtrate of blindworm bacilli. Controls using broth and peptone solution as antigen were negative.

Much and Leschke,⁴ using the serums of two goats injected with human tubercle bacilli, got fixation with emulsions of human tubercle bacilli, urine bacilli, blindworm bacilli, timothy hay bacilli, leprosy bacilli (from anti-forminized leprous skin), tuberculin and urine bacilli broth filtrate, but negative tests with blindworm bacilli broth filtrate and plain broth.

Deilmann⁵ studied human serum with emulsions of tubercle bacilli, urine bacilli, timothy hay bacilli, blindworm bacilli, leprosy bacilli (from antiforminized nodules) and tuberculin. Complement fixations were made in 239 persons ill with various diseases. In a large percentage of these cases there was no clinical tuberculosis nor tuberculous history. About 50% (118) reacted to one or more of the antigens used and of the positive reactions 84.6% were with tuberculin. With tubercle bacilli emulsion only 69.5% reacted positively. A few cases (15) were tested with lepra bacilli and 33% gave positive fixations. Many of the cases were tried with the blindworm, timothy hay and urine bacilli antigens, and small percentages of positive reactions were found although six cases gave reactions with urine bacilli and not with either of the tubercle antigens. Of the 118 cases in the series that gave positive reactions, only 11 were clinically tuberculous. He concludes that the tubercle bacillus contains specific complement binding substances in common with other related acid-fast bacilli. He also states that the complement binding substances present in human serum against tubercle antigen have little relation to clinically demonstrable tuberculosis but only indicate a previous contact tuberculosis. Against this last conclusion two important objections may be urged: First, that he used no control non-acid-fast antigens, and second, that the relatively large amounts of antigen used in each test—from 0.66 to 3.3 mg.—might lead to non-specific fixations, since control cases of other diseases were not included.

² Gengou has since privately stated that the potato medium employed in growing the antigens was probably itself antigenic and accounted for the cross reactions. (Information furnished by F. P. Gay.)

³ Beitr. z. Klin. d. Tuberk., 1910, 17, p. 199.

⁴ Ibid., 1911, 20, p. 351.

⁵ Ztschr. f. Immunitätsforsch. u. exper. Therap. (Orig.), 1911, 10, p. 421.

Using the same antigens as Deilmann, Wills⁶ tested 18 serums that had given positive reactions with the tuberculin antigen and got positive fixations with tubercle bacilli in 83%, leprosy bacilli in 27%, urine bacilli in 27%, timothy hay bacilli in 11%, and blindworm bacilli in 5%. All the antigens, with bovine tubercle bacillary emulsions also, gave positive fixation in more than half of 11 cases of leprosy tested.

Edith Claypole,⁷ in her classification of the streptothrix group of bacteria on the basis of fixation, found not only group reactions between acid-fast members of the streptothrices, but between them and the tubercle bacillus on the one hand and the non-acid fast streptothrices on the other. By means of variable amounts of antigen, however, relative differences between homologous and heterologous strains and a given immune serum could be shown. This work of Claypole's and her method formed the point of departure in our own study to be herewith presented.

Kritchewsky and Bierger⁸ report that the serums of a rabbit and a monkey immunized with Kedrowsky's leprosy bacillus gave fixations with human tubercle bacilli and Duval's chromogenic leprosy culture somewhat weaker than the homologous antigen. Antigens of Korn's bacillus I and *B. typhosus* did not react with the serum. Tests of four rabbits injected with Kedrowsky's culture were negative with Duval's culture although giving good fixation with the Kedrowsky antigen. One of these serums when tested with human tubercle bacilli gave a positive reaction. Two other rabbits immunized with Duval's culture, while reacting well with the homologous antigen, were negative with Kedrowsky's⁹ culture.

Harris and Lanford¹⁰ immunized rabbits against *B. smegmatis*, timothy hay bacillus, *B. tuberculosis* (avian) and the acid-fast organisms isolated from lepers by Kedrowsky, Bayon, Clegg, Currie and Duval (chromogenic and nonchromogenic strains). They tested these serums with antigens made from these organisms, and the butter bacillus and dung bacillus. In addition the serum of a rabbit injected with bacilli from a leper nodule was used in some of the tests. The organisms were grown on glycerol agar and the antigens used were 2% emulsions in salt solution of the alcohol precipitated bacterial growth. With decreasing doses of the various antigens in cross fixations until the minimal fixing dose was reached, an attempt was made to differentiate the several organisms. Cross fixation, however, was almost universal, and it was not uncommon that the minimal fixing dose of homologous antigen with an antiserum was higher than the minimal fixing dose of heterologous antigen. They concluded that their fixation tests showed no clear-cut specificity for any of the acid-fast bacilli studied.

Although these reports of previous investigators show the presence of a well-marked group reaction, including many of the acid-fast organisms, with consequent difficulty in classifying this group of organisms by complement fixation, a further attempt to use this method as a basis of grouping has been made. The first series of tests differed considerably from later experiments, and will be considered separately.

⁶ *Centralbl. f. Bakteriol., O., I*, 1912, 61, p. 37.

⁷ *Jour. Exper. Med.*, 1913, 17, p. 99.

⁸ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1913, 73, p. 509.

⁹ *Ibid.*, 1901, 37, p. 52; 1910, 66, p. 1.

¹⁰ *J. Infect. Dis.*, 1913, 13, p. 301.

METHODS

Antigens.—The acid-fast bacilli used were cultures of the following organisms: the strains of *B. leprae* of Duval¹¹ (chromogenic and nonchromogenic), of Bayon, of Brinckerhoff,¹² of Kedrowsky,⁹ of Clegg,¹³ of Levy¹⁴ and Karlinsky;¹⁵ *B. tuberculosis*, human and bovine types; *B. smegmatis*, butter bacillus (Rabinowitsch), and dung bacillus (Moeller No. 1), and *B. Lombardo Pellegrino*¹⁶ (a pigmented saprophyte). All cultures except those of *B. tuberculosis* were obtained from Dr. C. W. Duval. Duval's¹¹ nonchromogenic strain of *B. leprae* was at first grown with difficulty but after several transplants grew well on glycerol agar. The cultures of *B. tuberculosis* were transplants from strains originally obtained from Dr. Theobald Smith.

A discussion of the growth characteristics and morphology of each of these bacilli will not be given here. The various organisms differed in their degree of "fastness" when treated with acids or with alcohol as all workers have found. The strain of *B. smegmatis* conformed to the usual description by being acid-fast but not alcohol fast.

The tubercle bacilli were cultivated on Dorset's egg medium for from 4 to 6 weeks. The remainder were grown on glycerol agar in large flat Blake bottles. When the growth was fairly abundant, which was at the end of from one to two weeks, it was scraped off and washed from the surface of the medium with as little salt solution as necessary. This bacillary emulsion was then treated with somewhat more than an equal volume of 95% alcohol when the protein was precipitated as a flocculent precipitate. After immediate rapid centrifugalization and the removal of the clear supernatant fluid, the bacteria were dried over sulphuric acid in partial vacuum and later pulverized. This bacterial powder was finely ground in an agate mortar and a 2% suspension in 0.85% salt solution with 0.5% phenol. These 2% bacterial suspensions were used as antigens.

In the tests a constant amount of serum was used, and progressively decreasing doses of antigen dilutions were made with 0.85% salt solution. One cc of 1:20, 1:40, 1:80, 1:160, etc., dilutions of the antigens representing 1, 0.5, 0.25, 0.125 mg. etc., respectively, of the dried bacterial protein were used.

Immune Serum.—Two rabbits were immunized with each antigen by 5 intravenous injections at 3-day intervals and bled 9 days after the last injection. Each intravenous injection was 0.5 cc of the antigen, i. e., 10 mg. of dried bacterial powder. The immune serum was inactivated 30 minutes at 56 C. and kept in sealed glass tubes. In each test 0.3 cc were used. Of the two serums obtained, the one which showed fixation with the highest dilution of the homologous antigen was used for the tests.

Hemolytic System.—This consisted of 4 units of a rabbit antiserum of 1:5,000 titer, 1 cc of a 5% suspension of sheep corpuscles and a fixed dose of 0.1 cc of fresh guinea-pig serum.

The test was made by incubating the immune serum and antigen with fresh solution at 37 C. for 1 hour in the water bath, adding the lysin and sheep cells, incubating again for 2 hours, and reading the results after allowing the

¹¹ J. Exper. Med., 1910, 12, p. 649; J. Infect. Dis., 1912, 11, p. 116.

¹² Pub. Health Rep., U. S. Mar. Hosp. Serv., 1910, 25, p. 1173.

¹³ Philippine J. Sc., 1909, 4, p. 403.

¹⁴ Gior. d. r. Soc. ital. d'ig., 1902, 24, p. 219.

¹⁵ Monatsh. f. prakt. Dermat., 1903, 39, p. 392; Allg. med. Centr.-Ztg., 1903, 72, p. 965.

¹⁶ Ann. d'ig. Sper., 1906, n. s., 16, p. 163.

tubes to stand over night in the icebox. The total volume of the test was 2.5 c.c. The usual controls for anticomplementary properties in antigen and serum were always used, and tests with every antigen and normal rabbit serum showed no nonspecific fixation in the dilutions used.

In testing a given immune serum successive decreasing doses of homologous antigen were used with a constant amount of serum until the smallest amount of antigen that gave fixation was reached. Cross fixations were then made with this serum and the other antigens in the same manner. Each immune serum, therefore, was used in cross fixations with descending doses of all antigens. In reading the results of the tests, considerable difficulty was encountered in determining the point at which sufficient inhibition occurred in a series of antigen dilutions to call the test positive. When larger amounts of antigen were employed there would be complete inhibition and as less and less antigen was used hemolysis would gradually increase until finally it was complete. For example, in testing a given serum with 1:20 and 1:40 dilution of antigen no hemolysis would result, in the 1:80 and 1:160 dilutions, hemolysis would be partial and in 1:320 dilution it would be almost complete. On account of these gradations, the readings were always made at the lowest dilution of antigen which showed complete inhibition of hemolysis. The anticomplementary properties of the different antigens were carefully determined and no fixation test was considered positive unless double the dose of antigen used was not at all anticomplementary.

Table 1 shows the results of the fixation with the various antigens and immune rabbits serums.

It must be remembered that the readings were made at the point where there was complete inhibition of hemolysis, and that those reactions marked "o" in the table do not mean that there was no evidence of cross-fixation, but that the fixation was not complete with half the antigen dose that was not at all anticomplementary. Indeed, there was in all these instances very definite partial cross-fixation. Several of the serums, including those of animals immunized with tubercle bacilli, were tested with only a few of the antigens. There is no indication, however, that the completion of the cross-fixations with these serums would have altered the general results obtained.

In studying the tabulated results of the tests it is quite evident that there is a widespread group reaction that includes all the acid-fast organisms studied and that any classification or grouping of the strains cannot be made by the use of the methods employed in this series. It will be noted that the *B. leprae* cultures of Duval (chromogenic) and of Levy, showed fixation in greater dilutions with the immune serums of these two organisms than with any of the other serums. This same grouping is also noted with Duval's¹¹ nonchromogenic strain and Kedrowsky's⁹ culture. The other cross-fixations reveal differences so slight and inconsistent that any effort at grouping based on these results

TABLE 1
COMPLEMENT FIXATION WITH IMMUNE RABBIT SERUMS SHOWING DILUTIONS OF ANTIGENS GIVING COMPLETE FIXATION WITH FIXED DOSE
OF 0.3 c c OF IMMUNE RABBIT SERUMS

Antigens (2%)	Largest Amount of Antigen Not Anticomplementary (Antigen Control)	Immune Rabbit Serums (0.3 cc)														
		Nor- mal Serum Con- trol	B. leprae Duval (Chrome)	B. leprae Bayon	B. leprae Brinek- erhoff	B. leprae Clegg	B. leprae Levy	B. leprae (Non- chrome)	B. leprae Kedrow- sky	B. leprae Karlin- sky	B. smeg- ma- tis	B. of Moeller	B. of Butter	B. Lon- bar- do Pel- legrino	B. tu- ber- cu- lo- sis (Hu- man)	B. tu- ber- cu- lo- sis (Bo- vine)
B. leprae—Duval (chrome).....	1-10	+	1-320	1-20	0	0	1-160	1-20	0	—	0	1-20	0	1-40	—	—
B. leprae—Bayon.....	1-5	+	1-20	1-80	1-20	1-20	1-40	1-20	1-20	—	1-40	1-20	1-20	1-80	—	—
B. leprae—Brinkerhoff.....	1-3	+	1-20	1-20	1-80	1-40	1-20	1-20	1-20	—	1-40	1-20	1-80	1-160	—	—
B. leprae—Clegg.....	1-5	+	1-20	1-20	1-20	1-20	1-20	1-20	0	—	1-40	1-20	1-80	1-40	1-20	—
B. leprae—Levy.....	1-3	+	1-320	1-20	0	1-10	1-160	1-20	0	—	1-40	1-20	1-40	1-40	1-40	—
B. leprae—Duval (nonchrome).....	1-10	+	1-40	1-40	1-40	1-40	1-40	1-80	1-80	1-20	1-40	1-20	1-40	1-40	1-40	—
B. leprae—Kedrowsky.....	1-10	+	1-20	1-40	1-20	1-20	1-20	1-10	1-20	—	1-40	1-20	1-40	1-20	1-40	—
B. leprae—Karlinsky.....	1-3	+	1-20	1-40	1-10	1-10	1-20	1-10	1-20	—	1-320	1-40	1-160	1-20	—	—
B. smegmatis.....	1-10	+	1-80	1-80	1-80	1-40	1-40	1-40	1-40	—	1-320	1-40	1-20	1-80	—	—
B. of dung—Moeller No. 1.....	1-5	+	1-20	1-40	1-10	1-10	1-10	1-20	1-10	—	—	1-40	1-20	1-20	—	—
B. of butter—Rabinowitsch.....	1-3	+	1-20	1-20	1-10	1-10	0	0	0	—	1-20	—	—	1-160	—	—
B. Lombardo Pellegrino.....	1-10	+	1-20	1-20	0	1-10	0	0	0	—	1-20	—	—	1-160	—	—
B. tuberculosis—human.....	1-5	+	—	1-10	1-10	1-10	1-10	1-20	1-20	—	—	—	1-10	1-10	1-40	1-20
B. tuberculosis—bovine.....	1-3	+	—	1-10	0	0	+	1-20	1-20	—	—	—	1-10	0	1-20	+
No antigen (serum control).....	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

1-1, 1-20, 1-40, etc., indicate 1 c c of the corresponding dilution of the stock 2% antigen; + shows complete hemolysis; 0 a negative result and — the test not performed.

would not be justified. Occasionally an immune serum will fix a heterologous antigen in greater dilution than the homologous antigen. Since certain important points relating to the cross-fixations by different members of this group were demonstrated in other tests, a more detailed discussion of this subject will be made later. One feature, however, may be mentioned here. In making the antigens from the dried bacterial powder, a considerable variation in the evenness of the emulsion was observed. Some of the strains gave an antigen in which the emulsion was composed of a uniform and apparently even suspension of bacterial powder in a fine state of division, while other antigens showed some coarser particles. The coarser particles could not be overcome by long, continued grinding of the powder in an agate mortar, and were probably due in some way to the fatty substances of these particular organisms. The importance of this variation in the antigen emulsions lies in the fact that the antigens which gave the best emulsification with salt solution were those which gave fixation in the greatest dilution with the various immune serums, while those antigens in which the particles were coarser showed fixation only in low dilutions. There was, therefore, a definite variation in the antigenic properties of the different antigens due to the fact that all could not be emulsified equally well in salt solution. This factor naturally interferes with any deductions drawn from the comparison of the amounts of the different antigens necessary for complement fixation with immune serums.

In examining the results obtained by this series of fixations, it seemed that the method of study used was open to several objections, or at least that certain rather fundamental improvements in the methods used would overcome some of the difficulties in the interpretation of the results. The determination of the least amount of antigen necessary to give fixation with a given serum, for example, is merely an antigen titration and does not necessarily indicate the concentration of complement-fixing substances in the serum. It would seem of much more value to titrate serum with a fixed dose of antigen and thereby determine the least amount of serum that would react with the various antigens. It would also be of advantage to test the specificity of the reaction by control cross-fixations with organisms of this group and unrelated nonacid-fast organisms. It is obvious that with such a widespread group reaction as shown in this series, a more delicate hemolytic system would contrast the reactions with different antigens more

sharply and offer a better chance for differentiation. Since the binding of a certain amount of complement is what constitutes a positive reaction, and the less fixation substances are present in a serum the less complement will be fixed, the test can readily be made more delicate by using as small an amount of complement as can be employed with safety. Also, the difficulty experienced in interpreting fixations that are incomplete can be greatly obviated by employing a weaker suspension of red cells in the hemolytic system. In all later work the methods used were altered and the improvements suggested by this first series were made the basis for a further study of fixation reactions with this group.

In the second series of experiments, three important variations were introduced as a result of the difficulties in the first series. These were: First, the use of a larger number of acid-fast bacterial antigens with the addition of several nonacid-fast organisms as controls; second, the titration of the concentration of the fixation antibodies in all immune serums with each of the antigens; and third, the use of a more delicate and accurately controlled hemolytic system in which relatively small amounts of complement were necessary. By the use of these methods, the experiments were considerably broadened, and several interesting features concerning fixation with immune rabbit serums and bacterial antigens, especially those belonging to the acid-fast group, have been studied.

Antigens.—The acid-fast organisms used as antigens included 11 strains isolated from lepers, the *B. leprae* of Duval¹¹ (chromogenic and nonchromogenic), of Levy,¹⁴ of Kedrowsky,⁹ of Karlinsky,¹⁵ of Bayon, and the cultures B, F, G, H and 18, isolated by Clegg, Currie, Brinkerhoff and Hollman;^{12, 18, 17} 4 cultures of saprophytic acid-fast, smegma bacillus, butter bacillus (Rabinowitsch), the dung and timothy bacillus of Moeller;¹⁸ 4 cultures of *B. tuberculosis*, human, bovine, avian and the "turtle" culture of Friedmann. The non-acid-fast cultures used as control antigens were *B. typhosus* (Rawlings strain), *B. coli*, *B. abortus*, and a diphtheroid bacillus isolated from a lymph node from a case of Hodgkin's disease. In addition, an emulsion of Hansen bacilli from lepromas was used as an antigen. The strains of *B. leprae* isolated by Clegg and his co-workers were obtained from the late Dr. D. H. Currie, the Friedmann culture from the New York Museum of Natural History, the strain of avian tuberculosis (Avian 216) from Dr. Krumweide of the New York Department of Health, the human and bovine tubercle bacilli from Dr. Harry Foster of the Cutter Laboratories, Berkeley, Calif., and the remainder of the acid-fast organisms from Dr. C. W. Duval of New Orleans.

¹⁷ Treas. Dept. Bull. 47, Pub. Health and Mar. Hosp. Serv. 1911; Lepra, 1912, 13, p. 71.

¹⁸ Deutsche med. Wchnschr., 1898, 24, p. 376.

The bacterial antigens were prepared in the same manner as those used in the previous series. Human and bovine tubercle bacilli were grown on Dorset's egg medium, the other acid-fast bacilli on glycerol agar and the non-acid-fast cultures on plain agar. The dried, powdered alcoholic precipitate of bacterial culture was prepared as described. Emulsions of these antigens were made in salt solution so that one cc of the emulsion was equivalent to one mg. of the dried bacterial powder.

The material containing the leprosy nodules was obtained at necropsy in a case of advanced nodular leprosy. Several portions of thickened skin containing large numbers of leprosy bacilli were cut in small pieces and digested with antiformin at 37 C. until the tissue was disintegrated. It was then centrifuged and the sediment washed several times in salt solution. The sediment had a definitely yellow color and microscopically consisted entirely of acid-fast bacilli. It was dried over sulphuric acid and the bacterial powder used as antigen in exactly the same manner as the organisms from cultures.

In preparing antigen for use, 20 to 30 mg. of the powder were weighed out and thoroughly ground in an agate mortar. A more even emulsion was obtained by moistening the powder with salt solution and thoroughly mixing by grinding as the additional amount of salt solution necessary to make one mg. per cc was added. This antigen emulsion was used both for immunization of animals and for the fixation tests. It was kept at low temperature when not in use and frozen if possible. The addition of phenol or tricresol as a preservative perceptibly increased the anticomplementary action so that no preservative was used and freezing was depended on to prevent contaminating bacterial growth. The anticomplementary powers of such emulsions increased very gradually and after several weeks it was necessary to prepare a new antigen emulsion from the dried powder. The anticomplementary dose of such antigens varied from 0.2 to 2 cc (or mg.) and was relatively constant in freshly prepared emulsions of any antigen.

Immune Serums.—Two rabbits of 1,800 to 3,500 gm. were immunized with each antigen. Before immunization the serum of each animal was tested for nonspecific fixation with the antigen and if obtained with less than 0.4 cc of serum, the animal was discarded and another used. Four intravenous injections of from 1 to 4 mg. were given at 3 day intervals and 3 days after the last injection a final intraperitoneal dose of 6 to 12 mg. The size of the dose was determined by the weight reaction of the animal to previous doses. Animals were bled from 9 to 11 days after the intraperitoneal injection. The serum was sealed in small tubes and inactivated at 56 C. For the cross-fixations with other antigens, only the serum having the highest titer with the homologous antigen was used.

The Hemolytic System.—Various forms of hemolytic systems have been used in experimental work but the technic used in these experiments has proved so satisfactory in many ways that it is given in slightly greater detail than is usually thought necessary. No claim is made for originating it, however, since others have probably used a very similar procedure. Its use in a very large number of tests has proved its reliability and its delicacy.

The system consists of 2 units of complement, with 2 units of hemolytic sensitizer and 0.5 cc of a 1% suspension of sheep corpuscles made up to a total volume of 2.5 cc. The various ingredients must be standardized carefully.

The 1% suspension of sheep cells is more satisfactory when measured by centrifuging in a graduated centrifuge tube rather than by a pipet. The sediment should be lightly packed.

Rabbit antisherp serum of moderately high titer is diluted so that one minimal hemolytic dose with 1 cc of 1% dilution of complement is contained in 0.1 cc.

Pooled fresh guinea-pig serum is diluted 1:100. The complement unit is usually found to be 0.5 cc of this 1% dilution.

A preliminary titration of hemolysin with 2 units of complement, and of complement with 2 units of hemolysin is made as follows: Tubes are set up with 0.2, 0.15, 0.1 and 0.05 cc of hemolysin with 1 cc of complement; 0.8, 0.6, 0.5 and 0.4 of complement with 0.2 cc of hemolysin. All tubes are made up to 2 cc and 0.5 cc of 1% sheep cells added. The usual controls of hemolysin, complement and cells alone are included. Readings are made after one-half hour in the water bath at 37 C. In the hemolysin titration all tubes should be completely laked except the one containing only 0.05 cc of the hemolysin in which the hemolysis is incomplete. The complement titration should show complete laking in the first 3 tubes, leaving the tube with only 0.4 of complement partially hemolyzed. The controls should show no hemolysis.

It is rather remarkable how constantly the unit of complement is found to be 0.5 cc in this titration whether serum from one guinea-pig or pooled serum from several animals is used, provided the serum is fresh. The only factor that may cause some variation is the corpuscle suspension. In case the corpuscle emulsion is considerably more than 1%—as, for example, when it is made up from a very tightly packed centrifuge sediment—the complement titration will show a unit of 0.6 or 0.7 cc. In this case the usual values will be found by a slight dilution of the red cell suspension. It is almost unnecessary to say that the same error will follow the use of partially hemolyzed blood containing broken-down cells. A much more important factor, however, is the variation in resistance of the red cells of different sheep, since the corpuscles of some animals are very definitely more resistant to the hemolysis than the cells of other animals. In all the work here reported, the corpuscles used were obtained from animals kept by the laboratory and trouble from this source was not encountered since animals whose corpuscles were resistant were discarded. More recently, however, in doing similar tests it has been necessary to use blood obtained from abattoirs and the finding of cells somewhat more resistant to hemolysis has been not infrequent. With such blood the titration seems to indicate a weak or poorly acting complement, but it is relatively easy to demonstrate that the fault practically always lies entirely with the corpuscles. In such a case, if other less resistant cells are not available, one can only accept the unit shown in the titration.

The Tests.—The inactivated serum from an animal immunized with one of the antigens was tested as follows: Gradually decreasing amounts of serum from 0.05 cc to 0.0001 cc were measured into a series of tubes. For convenience and accuracy this was measured from dilutions of 1:10, 1:100 and 1:1,000. The antigen which had been previously titrated to determine the anticomplementary dose, was then added in less than one-half the dose that was not at all anticomplementary. As a rule, the amount used was 0.1 cc which corresponds to 0.1 mg. of the bacterial powder, and in all cases from 4 to 8 times this amount was not anticomplementary. After adding two units of complement (1 cc of a 1% dilution) and sufficient salt solution to make a total of 1.8 cc, the tubes were incubated in the water bath at 37 C. At the end of one hour 0.5 cc of 1% sheep cells sensitized with two units of hemolytic serum (0.2 cc) was added and the tests incubated one-half hour longer. Readings were made after standing over night in the icebox. The

usual controls of antigen alone and serum alone were always added to each series. Such a titration was made with every immune serum, not only with its corresponding antigen, but with all the antigens used.

In reading the tests the point at which complete inhibition ceased was always fairly sharply defined, and little effort was made to distinguish degrees of hemolysis. The standard adopted for reading titrations was complete inhibition or a very faint tinging of the supernatant fluid. The amount of serum in the last tube showing such an appearance indicated the minimal fixing dose of the serum with that antigen. The point at which inhibition stopped was so sharply defined that the result was never in doubt and the personal equation in reading the tests can be disregarded.

Time of Fixation.—The optimum time for the fixations was determined by finding the time at which the minimum amount of serum would give complete fixation with antigen; or, in other words, by finding the optimum incubation necessary for recognition of the smallest amount of complement-fixing antibody with antigen. For this purpose, the minimum fixing dose of serum with antigen was determined after incubating separate sets of tubes in the water bath at 37 C. for periods of 30, 45, 60 and 75 minutes; in the air bath for the same periods; and at 8 C. (icebox) for 4 hours. It was found that smaller amounts of serum gave fixation after water-bath incubation than after air-incubation during the same period. Almost always tubes incubated in the water bath for one hour showed a higher titer after one hour than for shorter periods, and this was not increased by leaving them 15 minutes longer. Incubation in the icebox (8 C.) for 4 hours showed also somewhat lower titer than after one hour in the water bath. Observations on a number of serums with several antigens constantly confirmed the fact that one hour in the water bath at 37 C. was the optimum incubation of those studied and consequently this period was adopted for all the tests.

Amount of Antigen Used in Tests.—By using the dried bacterial powder prepared as described, it was possible to use constant and comparable amounts of antigen in tests carried out at various times with the same or different immune serums. The question of the proper amount of antigen to use in the test was one which required some study. Two points in particular seemed necessary to determine: first, whether an increased amount of antigen above several minimal fixing units would increase the sensitiveness of the fixation test, i. e., if one has several times the amount of antigen sufficient to combine with a fixed amount of alexin and a certain quantity of fixation antibody, will an increase in the amount of antigen cause fixation with a still smaller quantity of immune body? Second, whether a relatively large amount of antigen has a tendency to cause a nonspecific fixation independent of any true anticomplementary action. The first of these two points was determined by finding the minimal fixing dose of an immune serum with an arbitrary dose of antigen, e. g., 0.1 mg. With three times this amount of serum, i. e., 3 minimal fixing doses, the minimal fixing dose of antigen, or antigenic unit, was titrated, and finally the serum was again titrated and the minimal fixing dose again determined, using 3 antigenic units. In this manner several different serums and antigens were studied and the results were quite uniform in indicating that if at least 3 units of antigen are used in titrating a serum, fixation will be obtained with quite as small amounts of the serum as if 10 or 20 antigenic units are used. It seems unnecessary to give more than a single example of such tests. The serum of a monkey dead from tuberculosis was titrated with 0.2 mg. of human tubercle bacilli antigen (of which 1 mg. was not anticomplementary) and the minimal fixing dose of serum found to be 0.004 c.c.

Using a fixed dose of 0.012 c.c. of serum, the antigen was titrated and the minimum fixing dose of antigen or antigenic unit was determined as 0.01 mg. A retitration of the serum using 0.03 mg. of antigen was identical with the original titration, the minimal fixing amount of serum being 0.004 c.c.

The tendency of an unusually large amount of antigen to cause nonspecific fixation with the immune rabbit serums quite independently of any anticomplementary action has been definitely indicated in the course of the experiments. It is most noticeable when doing cross-fixations with highly immune serums and heterologous antigens, and for this reason care must always be exercised in the amount of bacterial antigen used. It is felt that the use of from 0.05 to 0.1 mg. of antigen in the tests here reported probably reduced this factor to a minimum. A more detailed discussion of this nonspecific fixation will be given later.

Controls.—In every series of tests the usual control tests for anticomplementary properties in the serums and in the antigens were set up using double the largest amount of these reagents alone. Every serum was shown to contain only a slight amount of natural hemolytic sensitizer. The fact that 0.4 c.c. of serum from each rabbit was tested before immunization and gave no fixation with antigen was considered a sufficient control for the absence of fixation with normal serum and a given antigen. A further control of normal rabbit serum with each series of tests was, therefore, omitted.

In order to illustrate the method by which the tests were made, the record of one typical immune rabbit serum will be given together with a table showing its reactions with several of the antigens. Other immune serums for each of the antigens were made and tested in the same manner. A later table summarizing the results of all the cross-fixations will be understood to have been compiled from a series of tests such as the one given as an example.

RABBIT 507.—Weight, 2,160 gm.; serum from a preliminary bleeding which was tested Nov. 13, 1916, with antigen *B. leprae* (Kedrowsky) showed no fixation in 0.4 c.c. The rabbit was given intravenous injections of the same antigen as follows: Nov. 14, 3 mg.; Nov. 17, 20 and 23, 4 mg., and on Nov. 28, 12 mg. intraperitoneally. On Dec. 9, the 11th day after the intraperitoneal injection, the animal was exsanguinated, the serum collected, sealed in ampules and inactivated.

TABLE 2
TITRATION OF FIXATION ANTIBODIES WITH VARIOUS ANTIGENS—RABBIT 507

Antigen 0.1 mg.	Amounts of Serum in c c										Control Antigen 0.2 c c
	0.2	0.008	0.006	0.004	0.002	0.001	0.0006	0.0004	0.0002	0.0001	
<i>B. leprae</i> —Kedrowsky.....	..	++	++	++	++	++	++	++	+	—	—
<i>B. leprae</i> —Duval (chrome)	++	+	±	—	—	—	—	—	—	—
<i>B. leprae</i> —F.....	..	++	++	++	—	—	—	—	—	—	—
<i>B. leprae</i> —H.....	..	++	+	±	—	—	—	—	—	—	—
<i>B. tuberculosis</i> —human....	..	++	++	++	++	++	++	++	+	—	—
<i>B. tuberculosis</i> —avian....	..	++	++	++	++	+	—	—	—	—	—
<i>B. of butter</i>	++	++	++	++	—	—	—	—	—	—
<i>B. of timothy</i>	++	++	++	±	—	—	—	—	—	—
No antigen.....	—										

++ = complete inhibition; + = very faint hemolysis; ± = almost complete hemolysis;
— = complete hemolysis.

Table 2 shows the results of fixation tests made with this serum March 17, 1917. Only a few of the antigens are given, although cross-fixations were made with all antigens as may be noted by referring to the larger table in which the complete results are given. The method of recording the tests in table 2 is as follows: “++” denotes complete absence of hemolysis, “+” denotes almost complete inhibition with very faint hemoglobin-tinging of the supernatant fluid, “±” denotes marked but incomplete hemolysis, and “—” complete hemolysis. The relatively weak cell suspension made such readings very sharp after standing 12 hours in the icebox. Both “++” and “+” were considered positive, while “±” and “—” were called negative.

Before taking up the completed results of the tests, a few observations on the persistence of the fixation antibodies in rabbit serum may be mentioned. One animal after immunization with *B. tuberculosis* (human) showed a fixation with 0.0006 cc of serum with *B. tuberculosis* antigen. This animal was bled again two months later and the serum then showed fixation with 0.03 cc when tested with the same antigen. This indicates that while such antibodies may persist for a considerable time, the concentration becomes much weaker. In this respect, they resemble other specific immune substances. Other observations relate to the persistence of such fixation bodies in serum which has been kept sterile in sealed tubes for long periods. Samples of three serums, which had been used for tests in the first series of experiments mentioned in this paper and had been kept at room temperature, were retested with the second series, from 3½ to 4 years later. Unfortunately, the original titer of these serums cannot be stated since they were examined only by antigen titrations, but antibodies in rather high concentration persisted in these serums after this relatively long period. These serums were from rabbits immunized with antigens of the strains of *B. leprae* of Duval (chromogenic and non-chromogenic) and of Kedrowsky. They showed fixation titers of 0.006 cc, 0.001 cc and 0.006 cc, respectively, with Duval's¹¹ chrome, Duval's nonchrome, and Kedrowsky's⁹ organisms, which were the homologous antigens. All three serums gave fixation with a number of other acid-fast antigens, but, as a rule, in somewhat larger amounts. This evident slow deterioration of such antibodies has some bearing on the present series of experiments. On account of the number of immune rabbit serums and antigens studied, it was not always possible to test each serum immediately and sometimes several months elapsed before all the cross-fixations were completed with a given serum. It was, therefore, gratifying to have evidence that any marked deterioration in the fixing properties of a serum was unlikely. The observations just cited, together with the tests in general, give evidence, also, that the dried bacterial powder retains its antigenic properties unimpaired for a number of years.

The results of all the cross-fixations are summarized in table 3, which gives the minimal fixing dose of each serum with the various antigens. For convenience, the serum amounts are expressed in cubic millimeters instead of cubic centimeters, e. g., 1=0.001 cc, 50=0.05 cc. A blank indicates that the test was not made and 0 means that 0.2 cc of serum gave no fixation. The other features need no explanation since they seem clearly indicated in the table.

At first glance, the cross fixation seems to be practically universal, not only with the acid-fast antigens, but also with the few nonacid-fast organisms studied. It will be noted, however, that, while a given serum may fix with a number of antigens, usually a relatively small

TABLE 3

FIXATION REACTIONS WITH IMMUNE RABBIT SERUMS AND ACID-FAST ANTIGENS; MINIMAL FIXING DOSE OF EACH SERUM EXPRESSED IN CUBIC MILLIMETERS

	Largest Amount of Antigen Not Inhibitory, in Mg.	Amount of Antigen Used in Each Test, in Mg.	Antigenic Unit in Mg.	Immune Rabbit Serum														Serum								
				B. leprae—Duvál (chrome)	B. leprae—Bayon	B. leprae—Levy	B. leprae—B	B. leprae—F	B. leprae—G	B. leprae—H	B. leprae—18	B. leprae—Hansen	B. leprae—Kedrowsky	B. leprae—Duvál (nonchrome)	B. tuberculosis—Human	B. tuberculosis—Bovine	B. tuberculosis—Avian	B. tuberculosis—Turtle	B. of dung—Moeller	B. leprae—Karilnsky	B. of timothy—Moeller	B. of butter	B. smegmatis	B. typhosus	B. diphteroid, Serum 1	B. diphteroid, Serum 2
B. leprae—Duvál (chrome).....	0.8	0.1	0.006	2	4	6	30	30	10	9	6	2	1	1	4	4	1	30	6	2	2	4	30	8	0	0
B. leprae—Bayon.....	0.8	0.1	50	0.6	4	6	30	30	8	4	4	4	4	4	4	4	1	4	2	4	30	6	6	0	0
B. leprae—Levy.....	0.6	0.1	4	4	2	1	10	30	8	4	4	2	2	2	2	2	30	30	2	2	4	30	30	0	0
B. leprae—B.....	0.8	0.1	8	4	4	4	30	6	2	4	8	4	1	4	4	4	4	8	4	4	8	8	8	0	0
B. leprae—F.....	2.0	0.1	0.02	6	4	4	1	2	4	8	4	10	4	2	4	2	6	8	10	4	4	8	6	8	0	0
B. leprae—G.....	1.0	0.1	0.05	6	4	4	1	1	4	30	4	30	4	1	4	4	4	6	10	8	4	8	6	8	0	0
B. leprae—H.....	0.8	0.1	0.04	8	6	8	1	2	10	30	6	30	4	1	4	4	4	6	45	30	4	6	6	6	0	0
B. leprae—18.....	0.4	0.1	0.05	100	8	30	10	30	4	100	8	30	4	2	0.4	1	1	1	8	4	2	10	4	4	0	0
B. leprae—Hansen (from skin).....	0.8	0.1	0.01	30	2	1	8	10	4	30	6	4	0.2	0.2	1	1	1	1	4	4	2	0.6	4	4	0	0
B. leprae—Kedrowsky.....	2.0	0.1	0.02	30	2	1	8	4	30	6	4	4	0.2	0.2	1	0.6	0.6	1	10	6	4	0.6	4	4	0	0
B. tuberculosis—human.....	1.0	0.1	0.01	30	4	1	30	8	4	30	8	4	1	4	0.6	0.6	1	1	30	6	4	1	30	0	0	0
B. tuberculosis—bovine.....	0.8	0.1	30	2	2	30	10	8	30	8	4	1	1	4	4	2	2	8	4	2	1	30	0	0	0
B. tuberculosis—avian.....	0.3	0.05	100	1	4	10	30	10	50	10	30	1	1	4	4	2	8	8	4	2	2	10	0	0	0
B. tuberculosis—turtle.....	0.6	0.05	100	0.6	6	6	30	8	30	8	30	4	4	4	6	2	4	2	8	4	2	10	0	0	0
B. of dung—Moeller.....	0.3	0.05	100	1	4	8	30	10	0	0	30	4	6	6	2	4	6	2	1	0.6	2	4	10	0	0
B. leprae—Karilnsky.....	0.4	0.05	50	1	6	8	30	8	50	30	30	4	6	6	2	2	4	2	2	2	1	6	10	0	0
B. of timothy—Moeller.....	0.2	0.05	100	1	10	8	30	10	100	30	30	4	4	4	6	2	8	1	2	0.3	2	6	30	0	0
B. of butter.....	0.6	0.1	0.04	10	2	2	8	8	4	30	6	4	1	4	2	1	4	8	4	2	2	2	10	30	0	0
B. smegmatis.....	0.6	0.1	10	1	2	8	8	4	30	6	4	1	4	2	1	4	1	8	4	2	0.6	1	6	30	0
B. typhosus.....	0.3	0.05	0	8	0	0	0	50	0	0	0	30	50	30	100	30	30	30	10	0	0	0.4	30	0	0
B. coli.....	0.2	0.05	0	8	0	0	0	0	0	0	0	30	0	30	0	30	30	10	0	0	0	0	30	0	0
B. abortus.....	0.2	0.05	0	8	0	0	0	0	0	0	0	30	0	50	0	50	0	30	0	0	0	10	30	0	0
B. diphteroid.....	0.3	0.05	0.02	8	0	100	40	0	0	0	30	30	30	0	30	0	30	0	0

0 means no fixation with 0.2 c.c.

number of antigens will give fixation with that serum near its titer limit with the homologous antigen. In other words, when a serum is titrated with a number of antigens, some will give fixation with a relatively small amount of the serum, while other antigens will fix only with considerably larger amounts. In order to make this more evident, the cross-fixations have been slightly rearranged in table 4. Here are shown only those antigens giving fixation with amounts of serum near the titer with the homologous antigen. The inclusion of only those antigens reacting with 5 times the minimal fixing dose of each serum with its homologous antigen has been arbitrarily made. The figures in the table indicate the multiples of that dose of serum necessary for fixation with any given antigen, e. g., 1 = the minimal fixing dose of serum for the homologous antigen, 2 = twice that amount, etc. *B. leprae* 18 has been omitted because of its poor antigenic properties.

In the tables a general group reaction including all the acid-fast organisms studied is conspicuous. Although, as has been pointed out, a given immune serum may fix well with only a moderate number of the antigens, and less well with the remainder, yet practically all the antigens give good fixation with a considerable number of the serums. Indeed, despite a few irregularities which will be discussed later, this group reaction appears sufficiently widespread and specific to deserve the name acid-fast fixation.

The attempt to classify the various organisms by grouping those antigens which gave the best fixation with the several serums has been made in table 4. Although an accurate differentiation and grouping of the acid-fast bacilli is rendered difficult by the general fixation affecting all members, nevertheless, a certain tendency to form subgroups can be detected clearly. This tendency is more striking in some cases than in others. The group of chromogenic leprosy bacilli, composed of the Duval,¹¹ Levy,¹⁴ B, F, G and H organisms, has a definite similarity in antigenic characters as shown by the cross-fixations both with the immune serums and antigens. This antigenic relationship is so well defined with the F, G and H strains that one is tempted to consider these organisms identical. It is significant that all three were isolated by the same observer (Clegg), two being from the Philippine Islands and the third from Hawaii. The Duval, Levy and B organisms seem to be more closely related to each other antigenically than to the other three strains. The remaining two chromo-

TABLE 4

FIXATION REACTION WITH IMMUNE RABBIT SERUMS AND VARIOUS ANTIGENS SHOWING ANTIGENS REACTING WITH FIVE TIMES THE MINIMAL FIXING DOSE OF EACH SERUM OF LESS

Immune Rabbit Serums													
B. leprae—Duval (chrome)	B. leprae—Levy	B. leprae—B	B. leprae—F	B. leprae—G	B. leprae—H	B. leprae—Hansen	B. smegmatis	B. leprae—Kedrowsky	B. leprae—Duval (nonchrome)	B. tuberculosis—Human	B. tuberculosis—Bovine	B. tuberculosis—Avian	B. tuberculosis—Turtle
B. leprae—Duval (chrome).....	4	4	2, 5	4	2	4	1
B. leprae—Levy.....	1	1	2	4	4	4	2	4
B. leprae—B.....	1	1	2	2	4	2	4
B. leprae—F.....	4	4	1	2	2	2	2	4	4
B. leprae—G.....	4	4	1	1	1	2	4	4	4
B. leprae—H.....	1	1	1	1	2	4	4	4
B. leprae—Hansen (from skin).....	2	1	1	3	..	2	4	4	4
B. smegmatis.....	1	1	4	4	1	1
B. leprae—Kedrowsky.....	1	1	1	4	1	2	2	2	1	1
B. leprae—Duval (nonchrome).....	1	1	1	4	1	5	1	1	1	1
B. tuberculosis—human.....	2	2	1	4	1	1	1	1	1	1
B. tuberculosis—bovine.....	4	4	1	4	1	1	1	1	1	1
B. tuberculosis—avian.....	4	4	1	4	1	5	1	1	1	1
B. tuberculosis—turtle.....	1	4	1	5	1	1	1	1
B. of dung—Moeller.....	4	4	4	3	5	..	4	4	2
B. leprae—Karlinsky.....	4	4	4	2	2
B. of timothy.....	4	4	4	2	2
B. of butter.....	4	4	1, 5	4	4	4	2	2
B. leprae—Bayon.....	4	4	4	2	2
B. typhosus.....	4	4	4	2	2
B. diptheroid.....	4	4	4	2	2
B. diptheroid, Serum 1.....	4	4	4	2	2
B. diptheroid, Serum 2.....	4	4	4	2	2

genic leprosy cultures studied appear unrelated to the others, one of them, 18, having very poor antigenic qualities and the other, Bayon's culture, falling in another group.

The remaining organisms form a large group distinct from the organisms just mentioned, but even here the tendency to form sub-groups can be detected. There is a somewhat more marked cross-fixation with those strains given last in the table (table 4) including the bovine, avian, and turtle tubercle bacilli, dung, timothy and butter bacilli, and the leprosy strains of Karlinsky¹⁵ and Bayon. Although this group is perhaps less well defined than the one including the chromogenic leprosy bacilli, their antigenic relationship seems distinct. Of the other organisms, four strains have the striking property of fixing well with a majority of the immune serums. These antigens are the nonchromogenic *B. leprae* cultures of Duval¹¹ and Kedrowsky,⁹ *B. tuberculosis* (human) and the smegma bacillus. This characteristic of the latter organisms is associated with a physical property of the antigens themselves, since these particular antigens could be suspended in salt solution better than the others. They gave a uniform antigen suspension free from clumps, and the bacterial protein appeared as an evenly distributed opacity. With the other antigens this even fine distribution of the antigen in suspension was disturbed by some coarser flocculi which it seemed impossible to break up, although, for the most part, the suspensions appeared finely divided. This inability to secure a uniformly even distribution of the antigen suspension was attributed to the fatty substances of the organisms. One strain, *B. leprae* 18, which seemed especially rich in waxy material, showed a constant tendency to form numerous coarse particles in the antigen suspension, and it is of interest that this organism showed the poorest antigenic properties of any of the acid-fast bacilli studied. In general, it was found that the organisms which gave the most even suspensions made the best antigens. The Hansen bacilli obtained from leper skin, while conforming to the general acid-fast fixation of the rest of the organisms, does not appear closely related to the cultures. Possibly certain other antigenic relationships may be indicated from the reactions, but they are somewhat indefinite. Even in the sub-groups indicated there are irregularities in the results with occasionally unexpected cross-fixations. These are unexplained and are possibly an evidence of the group reaction.

The relation of the nonacid-fast organisms used as controls to the group of acid-fast antigens is striking. The bacteria used, *B. typhosus*, *B. coli*, *B. abortus*, and a diphtheroid bacillus were chosen merely as examples of common nonacid-fast organisms. The fact that diphtheroid bacilli have been isolated from lepers and are considered by some observers (Kedrowsky) related to the acid-fast leprosy organism, influenced the selection of a member of this group. The tests with these antigens show a very interesting and striking nonspecific fixation. Many of the rabbit serums showed fixation with one or more of the nonacid-fast antigens, but always with relatively large amounts of serum. This difference in titer of a serum with the nonacid-fast and the acid-fast antigens, particularly that of the homologous organism, is very marked. Usually from 30 to several hundred times the minimal fixing dose of a serum with the homologous antigen is necessary to obtain fixation with a nonacid-fast strain. Also, all serums which gave fixation with the nonacid-fast organisms were of relatively high titer while the low titer serums failed to give such fixation.

That this nonspecific fixation is a rather general phenomenon is indicated by the fact that, not only can fixation be obtained with nonacid-fast antigens and rabbit serums highly immunized against acid-fast strains, but a highly immune antityphoid serum or antidiphtheroid serum will also give fixation with acid-fast and other antigens. The antityphoid serum tested in table 3 had an agglutination titer of 1:10,000 and gave fixation with all other antigens tested, although only in relatively low dilutions. A good example of the effect of the titer of an immune serum on nonspecific fixation with other antigens, is given in the results of two serums of different titer from rabbits immunized with the same antigen. In table 3 are shown the results of fixation tests with two serums immunized with a diphtheroid bacillus, one having a titer of 0.006 c c with the homologous antigen, and the other of 0.0006 c c. With the high titer serum, a nonspecific fixation is obtained with a number of heterologous antigens, although in only relatively low dilution, while with the serum of lower titer, fixation is not shown with other antigens. In every case nonspecific fixation occurs only with the serums of relatively high titer, and with many times the minimal fixing dose of such serums with the homologous antigens.

In the presence of such nonspecific fixation with bacterial antigens, the question arises of the relation of this phenomenon to the group fixation with the acid-fast organisms, and whether this group reaction may not be another evidence of nonspecific fixation. From one point of view it is certainly nonspecific, since frequently the organisms of this group have marked cultural, pathogenic and other biologic differences, but it is likewise evident that, as a group reaction, it may be considered specific for this group of organisms as distinguished from the nonacid-fast strains. In certain instances, when an acid-fast antigen gives fixation with an immune serum only in rather low dilutions, it seems probable that such fixation is an example of the general nonspecific fixation. A very large proportion of the cross-fixations with the acid-fast antigens, however, show fixation with amounts of serum that approach the titer limit of the serums. This high degree of cross-fixation does not occur in the usual nonspecific fixation noted and indicates a group reaction distinct from the nonspecific variety.

The cause of nonspecific fixation such as has been illustrated in this series of experiments is not altogether clear. It seems certain that anticomplementary action of the antigens or immune serums can be excluded. Two units of complement with amounts of antigen and serum that were not anticomplementary in four times the dose used, together with the usual controls, seem to make this explanation impossible. The method of preparation of the antigens, with the possibility of the introduction of substances such as small amounts of culture mediums or products of culture autolysis, might conceivably be concerned in the production of a nonspecific fixation. This factor has been controlled by several experiments, unnecessary to give in detail, in which both acid-fast and nonacid-fast washed organisms were used as antigens with the immune serums in similar fixation tests. Several rabbit serums immunized with washed bacteria were also tested with the antigens used in the larger series. In all instances, no differences in the nonspecific fixation were noted that could be attributed to the method of antigen preparation. It seems likely, therefore, that the bacterial antigens themselves contain the substances that are concerned in the production of the nonspecific fixation.

The tendency of immune serums of high titer to give such nonspecific fixation with other antigens as has been mentioned suggests that a certain amount of care should be used in all cross-fixations with high titer serums to avoid being misled by the general reaction. It is

felt that the somewhat more delicate method of performing the tests used in these experiments has made nonspecific fixation more apparent, but there is no reason to believe that it could not be demonstrated by other methods with the amounts of serum ordinarily used, e. g., 0.1 c c.

That the part played by the lipoid material of the acid-fast organisms in the fixation test may be important is indicated by the fact that ether extracts of tubercle bacilli and other acid-fast organisms may act as antigen in tests with immune serums, or with serums from patients suffering from such acid-fast infections as tuberculosis and leprosy. The work of Much^{3,4} and his collaborators^{5,6} with the so-called "partial antigens," while rather unconvincing in many respects, nevertheless illustrates this phenomenon. Lucke's¹⁹ experiments with tubercle wax antigen, also, show that the lipoid material from tubercle bacilli acts as antigen in fixation tests with serums of certain tuberculous patients. All such experiments have shown a rather high percentage of nonspecific fixations, possibly due to the relatively large dose of antigen employed in the tests. Such antigens are much inferior also to whole culture antigens in the number of positive tests obtained with serums of tuberculous individuals. In the present work no study of such antigens was made except to show that ether extracts of several dried antigens gave fixation with some of the immune rabbit serums. It is believed by some writers that fixation with such antigens is due merely to general lipotropic properties in certain serums since a rather large proportion of cases of syphilis give a positive reaction. None of the rabbit serums used in the present experiments, however, showed fixation even in amounts of 0.2 c c with a simple lipoid (Wassermann) antigen. With the present evidence it does not seem justifiable to regard the fixation test with immune rabbit serums and acid-fast antigens a purely lipotropic phenomenon. The presence of relatively large amounts of waxy or lipoidal material is one of the striking characteristics common to all the members of the acid-fast group, and it is reasonable to suppose that they are concerned in the group reaction. Even assuming that their part in the process is essential and that the fixation depends on their presence does not alter the specificity of the reaction for this group since other bacteria contain proportionately small amounts of lipoid material. It seems appropriate, therefore, to employ the term acid-fast fixation.

¹⁹ J. Immunol., 1916, 1, p. 457.

Another possible explanation for such nonspecific fixation is that it is an evidence of a general sensitization to bacterial protein which occurs in serums of highly immunized animals. Such a sensitization might even include any foreign protein. Since no effort has been made in these experiments to demonstrate a general sensitization of this nature, its possibility may be merely mentioned.

SUMMARY

In serums of rabbits immunized with various acid-fast bacteria there is present a complement-fixing antibody which reacts with all members of the acid-fast group examined. When such antibodies are studied quantitatively by titrating each immune serum with the different antigens in the fixation test, certain members of the group show a close antigenic relationship, and tend to form subgroups. Certain organisms of the group are less closely related, but cross-fixation is specific for this group of bacteria and may be termed an acid-fast fixation.

Some of the acid-fast bacterial antigens are constantly superior to others in giving fixation with high dilutions of all antisera obtained with members of this group, and these antigens are those that form even fine suspensions in salt solution. Those antigens with much waxy material tend to form suspensions containing coarser particles, and have inferior antigenic properties.

Control nonacid-fast bacterial antigens and rabbit serums immunized with such organisms were compared with the acid-fast antigens and their immune serums. Nonspecific fixation is obtained with nonacid-fast antigens in rabbit serums of high fixation titer immunized with acid-fast strains. Such fixation, however, is found only with relatively large amounts of antigen or serum. Similarly, acid-fast antigens give fixation in low dilutions only, if at all, with immune serums from nonacid-fast strains. This nonspecific fixation occurs only in immune serums of high titer, and, when present, is obtained only with many times the minimal fixing dose of serum with the homologous antigen. It is distinct from the group acid-fast fixation, and may be an evidence of sensitization to bacterial proteins in all highly immunized serums.

COMPLEMENT FIXATION WITH ACID-FAST BACTERIA

II. IN LEPROSY

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During the past decade, the study of leper serums with various antigens by the fixation test has interested a number of observers in various places. A correct comparison of the results of the many observations is rendered difficult because, not only has the technic of the reaction varied, but the kind of antigen used in the tests has been modified in numerous ways. In general, the recorded observations may be divided into three groups: (1) Wassermann reactions, (2) complement fixations with the use of tissue extracts, and (3) tests with bacterial antigens. It is rather striking that no matter what kind of antigen has been used, a certain proportion of positive results has been obtained. Some have reported a high percentage of positive tests, while others have obtained only a relatively small percentage. Apparently, all have felt that fixation antibodies are present in the leper serum, and the variety of antigens employed is an evidence of the search for the one that would constantly react with all or a large proportion of the cases.

From time to time partial and frequently inaccurate compilations of the work of investigators in this field have been made, but no comprehensive collection or analysis of cases occurs in the literature. Since it was found possible in the course of this investigation to review all of the original publications, it was thought desirable to tabulate the results of this study which comprises all the previously reported work on fixation in leprosy. Some care has been taken to make the bibliography complete, and it seems certain that this report contains all the reactions reported with the possible exception of certain isolated cases included with work not closely related and consequently extremely difficult of access. An additional reason for tabulating the cases is to illustrate the relation of my own studies to those of other investigators and to point out some rather fundamental features in fixation reactions with leper serums that have apparently not been

understood previously. In some instances a regrouping of the cases reported by certain authors has been rendered necessary by the arrangement adopted in the tables. Occasionally, I have taken the liberty of making certain slight alterations in authors' figures that seemed quite justified. For example, serums that were anticomplementary have been omitted entirely even though they had been included as "positive" by the author. Again, cases called "weak positive" have been included under positive while those reported as "very faint inhibition," "very faintly positive," "almost complete hemolysis," or "doubtful" have been placed with the negative cases.

One criticism that applies to a very large part of the work is the lack of proper controls. With regard to the Wassermann reactions this omission is probably less important, since this test has been performed on such a large scale on so many diseases. Very few authors made any but casual mention of any series of control serums tested, although it is possible that such omissions were merely due to the fact that it was not thought necessary to mention them. With regard to the other antigens, however, one can only assume that the very few control serums mentioned were the only ones tested. Obviously, the specificity of the reaction should have been controlled, not only by the use of a considerable number of serums from diseases other than leprosy, but also by the use of other antigens prepared from unrelated tissues or bacteria. This procedure has been carried out by only a very few.

THE WASSERMANN REACTION IN LEPROSY

The antigens used in Wassermann reactions have included alcoholic extracts of human and guinea-pig heart, normal and syphilitic liver, syphilitic fetus and the acetone insoluble lipoid antigen of Noguchi. Since it is generally agreed that these various methods of preparing lipoidal antigens have little effect on the result of the reactions, no attempt has been made to group the cases according to the antigen used.

The presence of fixation antibodies in leper serum with the various lipoid antigens used in the Wassermann reaction has been rather a pathologic curiosity. As may be seen by table 1, 42 investigators have made more or less extended studies on this phenomenon. One or two writers have been interested to determine whether leprosy causes a positive Wassermann reaction or whether a relatively high percentage of lepers have syphilis, but unfortunately there are few reported studies of unselected control groups of cases without leprosy to determine the percentage of latent syphilis. As a rule, however, the number of lepers giving a positive Wassermann is high enough to justify the conclusion that leprosy alone may cause the blood to contain substances that give fixation with lipoidal antigens. That the proportion of leper serums giving a positive Wassermann test is probably not so great as is generally considered is indicated by a summary of all the reported cases. In all there

TABLE 1
 WASSERMANN REACTION IN LEPROSY

Name of Author	Year	All Cases Leprosy		Nodular and Mixed		Anes- thetic Cases		Remarks
		Num- ber	Per Cent. Posi- tive	Num- ber	Per Cent. Posi- tive	Num- ber	Per Cent. Posi- tive	
1. Eitner.....	1908	1	100					
2. Wechseimann.....	1908	1	100	Fixation also with 1 per cent. lecithin
3. Slatinéanu and Daniélopou	1908	21	76	Spinal fluid gave positive reaction in 6 cases
4. Masslakowetz.....	1908	16	56	Controls: 2 syphilitics positive, 1 normal, negative
5. Jundell, Almkvist and Sandman	1908	23	35	12	42	11	27	2 other cases anticomplementary; 1 case suspected leprosy negative
6. Bruck and Gessner	1909	10	50	7	71	3	0	
7. Castelli.....	1909	1	100					
8. Ehlers and Bourret	1909	5	60	39 other serums were partially anticomplementary
9. Eliasberg.....	1909	50	56	31	81	19	16	
10. Fleming.....	1909	2	100					
11. Frugoni.....	1909	4	75	1 positive case has clinical syphilis
12. Inouye, Ito and Nakano	1909	39	58	23	10	16	31	
13. Noguchi.....	1909	10	70					
14. Serra.....	1909	17	76	14	93	3	0	
15. Akerberg, Almkvist and Jundell	1910	14	15	5	40	9	0	Supplementary report to (5)
16. Babes and Busila	1910	9	89	8	100	1	0	Used 0.2 to 0.8 c.c. of serum
17. Baermann and Netter	1910	140	56	124	65	16	50	In 100 normal (Chinese) controls 25 per cent. positive
18. Fox.....	1910	60	57	38	82	22	19	
19. Meier.....	1910	31	45	19	63	12	17	
20. Montesano and Sotiriades	1910	48	48	36	86	12	17	
21. Nishiura.....	1910	136	32	67	46	69	19	
22. Steffenhagen.....	1910	5	60					
23. Thomsen and Bjarnhjedinson	1910	50	22	31	36	19	0	53 per cent. were positive with active serum
24. Bloomberg.....	1911	21	14					
25. Duval and Gurd..	1911	27	81	22	91	5	40	
26. De Haan and Grijns	1911	18	39	6	33	12	42	2 of the negative cases were "doubtful"
27. Krefling.....	1911	1	100	Negative with another antigen
28. Müller and Suess..	1911	6	100	6	100			
29. Schmitt.....	1911	7	71	5	80	2	50	
30. Bates.....	1912	42	47					
31. Photinos and Michailides	1912	204	56	104	75	100	38	
32. Raemora.....	1912	19	74	17	82	2	0	Salvarsan had no effect on reaction
33. Schöffner.....	1912	32	26	32	26			
34. Wills.....	1912	10	40					
35. Clegg.....	1913	24	46	All positives were nodular type
36. Kritehewsky and Bierger	1913	17	71	11	100	6	17	
37. Lagane and Columbler	1913	7	71	3	67	4	75	
38. Schmitter.....	1914	25	80					
39. Fletcher.....	1915	100	22	44	27	56	18	10 per cent. of 110 cases malarial and beriberi positive
40. Mathis and Baujean	1915	41	2	40	2	1	0	Positive case had clinical syphilis
41. Nakajo and Asakura	1915	23	69	18	74	5	60	
42. Sordelli and Flecher	1916	80	61	1 case anticomplementary not included; 103 cases not leprosy gave 13 positives
Totals.....	1,397	50	723	60	405	25	

have been collected 1,397 cases of leprosy of which 705 or 50.4% have given a positive reaction. The general impression that one gets from statements in textbooks and articles discussing the Wassermann reaction in leprosy is that the percentage of positive results is considerably higher than this figure, although no previous analysis of such a large series has been attempted.

The relation of the type of the disease to the presence of a positive reaction may be determined by an analysis of those cases in which the observer classified the cases studied. In this, cases of nodular or skin leprosy have been grouped with those having evidences of the disease both in the skin and nerves—the so-called mixed type—and the purely nerve or anesthetic cases have been grouped separately. Seven hundred and twenty-three cases of nodular and mixed leprosy have given 433 positive reactions, or 60%, while of the 405 cases of anesthetic leprosy, only 103, or 25.4%, were positive. That these figures are comparable to the large group of all the reported cases is shown by combining them—1,128 cases with 536, or 47.5%, positive.

To summarize approximately these results, it will be seen that half of all the cases of leprosy studied have given a positive Wassermann reaction, while three fifths of the nodular and mixed cases and one fourth of the purely anesthetic type have reacted positively. Since this fixation is a lipoidal reaction and admittedly nonspecific, no discussion of its significance will be made here. It is obvious, however, that while a certain proportion of the positive fixations may be accounted for by a coincident syphilitic infection, the percentage is entirely too high to be explained by this factor alone, and we must conclude that infection with the Hansen bacillus may cause the appearance in the blood of substances capable of giving a positive Wassermann reaction, and that these substances are more frequently present in cases with skin lesions, than in cases with nerve lesions alone.

TISSUE EXTRACT ANTIGENS

In reviewing the results of complement fixation in leprosy with antigens made from tissue extracts, two groups have been made: (1) alcoholic, and (2) aqueous extracts of tissues. The first group is tabulated in table 2. The preparation of these antigens is essentially similar to that used for Wassermann antigens except that lepromas were extracted by alcohol and in a few instances the ether soluble substances of such tissue were used also. Such antigens are evidently essentially lipoidal and their use should give results similar to those obtained in the Wassermann series. By referring to the table, it will be seen that of the 210 cases tested with these antigens 110 or 52% were positive. In almost all of these the type of disease is stated and 82 (75%) of the 109 tubercular and mixed cases, with only 23 (25%) of the 91 purely anesthetic cases were positive. Even this relatively small series shows a rather close relation to the percentages found by using the usual lipoidal Wassermann antigen (table 1), and there seems no doubt that fixation with leper serums and alcoholic extract of leproma as antigen has no more specificity than the Wassermann reaction in similar cases. This is further shown by the work of **Frugoni** and **Pisani**, who obtained somewhat better

results with the use of alcoholic extracts of sarcoma and carcinoma as antigen than with the leproma extract.

TABLE 2
COMPLEMENT FIXATION IN LEPROSY WITH TISSUE EXTRACT ANTIGENS (ALCOHOLIC)

Author	Year	Antigen Alcoholic Extract of	All Cases Leprosy		Nodular and Mixed		Anesthetic Cases		Remarks
			Num-ber	Per Cent. Positive	Num-ber	Per Cent. Positive	Num-ber	Per Cent. Positive	
1. Babes and Busila*	1909	Lepromas†	5	100	5	100	1 of 3 cases of syphilis was positive 3 cases of syphilis negative
Babes and Busila	1909	Normal skin†	5	0	5	0	
2. Frugoni and Pisani*	1909	Lepromas	10	50					
Frugoni and Pisani	1909	Sarcoma	11	73					
Frugoni and Pisani	1909	Carcinoma	11	64					
3. Récio.....	1909	Lepromas	18	78	14	92	4	25	1 of anesthetic cases "doubtful"; 2 cases syringomyelia negative
4. Nishiura.....	1910	Leprous liver	135	44	67	64	68	24	4 of 10 cases of syphilis were positive
5. Schmitt.....	1911	Rat leprosy tissues‡	7	100	5	100	2	100	Of 5 cases of syphilis 3 were positive; 7 cases various diseases neg.
6. Jeanselme.....	1912	Alcoholic ether extract of lepromas	9	56	5	80	4	25	1 normal person and 2 out of 8 syphilitics gave positive reactions
7. Jeanselme and Vernes	1915	Same	7	57	4	100	3	0	32 of 40 cases of syphilis were positive; 21 normal persons negative
8. Nakajo and Asakura	1915	Lepromas	19	58	9	89	10	30	
Totals.....			210	52	109	75	91	25	

* Only tests with leproma antigen included in totals.

† Ether extract used.

‡ Acetone insoluble fraction (Noguchi).

The aqueous tissue extracts used as antigen were made by grinding fresh or dried lepromas with salt solution, and allowing the coarser particles to sediment. The supernatant slightly turbid fluid was preserved with a small amount of phenol or other antiseptic and used as antigen after testing its anticomplementary properties. Any variation of this technic used did not essentially alter the character of the antigen with the possible exception of two observers who used antiforminized lepromas as antigen. The results with such extracts are given in table 3. Two hundred and three or 66% of the 309 cases of leprosy gave positive reactions. One hundred and forty-four cases of nodular or mixed leprosy showed 121 or 84% positive while 56 or 41% of the 137 cases of the anesthetic type reacted positively. Among the relatively few control cases of other diseases tested with these antigens, certain cases of syphilis and of tuberculosis have reacted positively. The probable explanation of these reactions will be discussed later.

TABLE 3
COMPLEMENT FIXATION IN LEPROSY WITH TISSUE EXTRACT ANTIGENS (AQUEOUS)

Author	Year	Antigen Aqueous Extract of	All Cases Leprosy		Nodular and Mixed		Anes- thetic Cases		Remarks
			Num- ber	Per Cent. Posi- tive	Num- ber	Per Cent. Posi- tive	Num- ber	Per Cent. Posi- tive	
1. Eitner.....	1906	Lepromas	1	100	1	100			
2. Eitner.....	1908	Lepromas	1	100					
3. Gaucher and Abrami	1908	Lepromas	9	100	8	100	1	100	16 cases syphilis and 8 cases syringo- myelia negative; 3 of 10 cases tuber- culosis positive
4. Slatinéanu and Daniélopou	1908	Lepromas	26	92	11 of the 19 cases gave positive re- action with spinal fluid
5. DeHaan.....	1909	Lepromas	16	9	5	20	11	9	11 of the cases gave "doubtful" reac- tions
6. Pasini.....	1909	Lepromas	3	67	3	67			
7. Serra.....	1909	Lepromas	17	88	14	100	3	33	
8. Serra.....	1909	Normal organs	17	76	14	98	3	0	Control
9. Sugai.....	1909	Lepromas	2	100	1	100	1	100	5 other cases anti- complementary
10. Akerberg, Jun- dell and Almk- vist	1910	Lepromas	28	34	12	67	16	13	11 of 17 cases syph- ilis were positive; 6 of 11 cases syph- ilis and gonorrhea with negative Wassermann gave positive reaction
11. Babes and Busila	1910	Lepromas	9	100	7	100	2	100	Used 0.2 to 0.6 cc serum
12. Nishiyura.....	1910	Lepromas	141	65	67	82	74	50	Slightly higher per- cent. of positive reactions with aqueous extract of leprous liver antigen
13. Steffenhagen....	1910	Antifor- minized lepromas	5	80	5	80			
14. Thomsen and Bjarnhjed- son	1910	Lepromas	19	16	3	100	16	0	Same results with normal skin anti- gen; 3 cases syph- ilis positive
15. Biehler and Eliasberg	1911	Antifor- minized lepromas	18	94	8	100	10	90	
16. Clegg, McCoy and Hollman	1914	Lepromas	13	85	10	90	3	67	4 normal controls negative; 1 of 2 cases pulmonary tuberculosis pos.
Totals.....	308	66	144	84	137	41	

The proportion of positive fixation, therefore, in all types of leprosy, is higher when aqueous extracts of lepromas are used than when alcoholic extracts of leprous or other tissues are employed as antigen. Such aqueous leprôma extracts, beside certain lipoidal and other substances probably giving a nonspecific fixation, must contain in addition enormous numbers of acid-fast bacilli since the skin

nodules of leprosy are usually filled with micro-organisms. It is quite likely that these Hansen bacilli add a definite specificity to the antigen and may be largely responsible for the higher percentages. The results of Steffenhagen and Biehler and Eliasberg, who obtained more than 80% of positive reactions by using antiforminized lepromas as antigen, support this view, since such an antigen contains less nonspecific tissue juices and relatively more acid-fast bacilli. On the other hand, Serra and Thomsen and Bjarnhjedinson obtained almost as good results with aqueous extracts of normal organs as with lepromas, although the latter observers' figures are unusually low.

TABLE 4
COMPLEMENT FIXATION IN LEPROSY WITH BACTERIAL ANTIGEN (TUBERCULIN)

Author	Year	All Cases Leprosy		Nodular and Mixed		Anesthetic Cases		Remarks
		Num-ber	Per Cent. Positive	Num-ber	Per Cent. Positive	Num-ber	Per Cent. Positive	
1. Slatinéanu and Daniélopou...	1908	19	58					
2. Frugoni and Pisanl.....	1909	10	20					
3. Babes and Busila.....	1910	9	89	7	100	2	50	
4. Meier, G.	1910	9	89	9	89			
5. Steffenhagen.....	1910	5	60	5	60			
6. Moellers.....	1913	32	63					
7. Nakajo and Asakura.....	1915	38	63	23	79	15	33	1 other case anti-complementary
8. Chijo and Asakura.....	1916	10	70					
9. Sordelli and Fischer.....	1916	48	83	4 other cases anti-complementary
Totals.....	180	68	44	84	17	35	

BACTERIAL ANTIGENS

The cases in which bacterial antigens have been used have also been divided into two groups: (1) those tested with tuberculin as antigen, and (2) those in which bacillary emulsions have been used. The first group is tabulated in table 4. Apparently Koch's "old tuberculin" was used in most instances. Of 180 cases so tested, 123, or 68%, were positive. The type of leprosy was mentioned in only about a third of the cases, 44 being of the nodular or mixed variety and 17 anesthetic; 84% of the former and 35% of the latter were positive. Tuberculin, then, seems to act as well, or possibly slightly better, than aqueous extracts of lepromas as antigen.

The use of bacillary emulsion antigens has been limited to a relatively small number of cases which are tabulated in table 5. Suspensions of acid-fast bacilli were used in every instance. Several investigators have used tubercle bacilli, while others have used strains of acid-fast organisms isolated from lepers, but there has been relatively little variation in the results obtained with the use of the several organisms employed. Currie and Clegg, for

example, tested the serums of four lepers with antigens prepared from several strains of acid-fast bacilli isolated from lepers, as well as extracts and emulsions of *B. smegmatis* and *B. margarin*, two saprophytic acid-fast bacilli. Their results were somewhat irregular, all organisms giving fixations of varying degree with all the cases tested and they concluded that they were unable to differentiate between the various acid-fast bacilli in this manner. Duval

TABLE 5
COMPLEMENT FIXATION IN LEPROSY WITH BACTERIAL ANTIGENS (BACILLARY EMULSIONS)

Author	Year	Antigen Cultures of Bacillus	All Cases Leprosy		Nodular and Mixed		Anes- thetic Cases		Remarks
			Num- ber	Per Cent. Posi- tive	Num- ber	Per Cent. Posi- tive	Num- ber	Per Cent. Posi- tive	
1. Frugoni and Pisani	1909	Tubercu- losis	11	73					
2. Mezonescu.....	1909	Rat leprosy	24	92	18	100	6	67	1 other case anti-complementary
3. Steffenhagen....	1910	Tubercu- losis	5	60	5	60	2 cases gave positive reaction with antigen of typhoid bacilli
4. Currie and Clegg	1910	Various acid-fasts	4	100	3	100			
5. Duval and Gurd..	1911	Leprae of Duval	27	85	22	95	5	40	7 normal serums gave negative reactions but 22 of 23 cases of syphilis gave positive reactions
6. Bayon.....	1912	Leprae of Kedrowsky	1	100					
7. Wills*.....	1912	Human tubercu- losis	9	67					
	1912	Bovine tubercu- losis	11	64					
	1912	"Urine"	9	56					
	1912	Blindworm	9	44					
	1912	Timothy grass	9	56					
8. Kritchewsky and Bierger*	1913	Human tubercu- losis	8	88	7	86	1	100	
	1912	Leprae of Kedrowsky	28	68	20	90	8	13	Using 0.8 cc, 5 of the negative serums became positive
	1913	Leprae of Duval	28	7	20	10	8	0	5 nodular and 2 nervous cases gave "feebly positive" reaction
	1913	Korn I	11	0	11	0	2 cases gave "feebly positive" reaction
	1913	Typhosus	11	0					
Totals.....			89	83	55	93	12	58	

* Only the tests using human tubercle bacilli antigen are counted in the totals.

and Gurd, using an acid-fast organism isolated by the senior author as antigen, obtained a large percentage of positive fixations in lepers and an equally large percentage in syphilitics, both groups giving a positive Wassermann test. In other words, the emulsion of acid-fast bacilli was indistinguishable in its antigenic action in syphilis and leprosy from the usual lipoidal antigens. Steffenhagen noted that 2 out of 5 cases of leprosy tested by him gave positive fixation using an emulsion of typhoid bacilli as antigen. Wills found that several

strains of acid-fast organisms acted almost equally well as antigen with leper serums, but Kritchewsky and Bierger conclude that Kedrowsky's strain of *B. leprae* is superior in its antigenic properties to the other organisms studied and were unable to obtain fixation in leper serum with a *B. typhosus* antigen.

On the whole, bacillary emulsions have somewhat better antigenic properties than the other antigens used in leprosy, although the group of cases studied in this manner is small.

In table 6, the totals of all the groups are summarized and the proportion of positive fixations indicated. It is rather striking to note the gradually increasing percentage of positive results with the different types of antigens, the Wassermann antigen giving the lowest percentage and the bacillary emulsion antigens the highest.

Certain features of the analysis of the results of complement fixation in leprosy with various antigens may be briefly stated.

TABLE 6
SUMMARY OF POSITIVE FIXATION TESTS IN LEPROSY; ALL ANTIGENS

Table	Antigen	All Cases Leprosy with per Cent. Positive	Total Skin Cases with per Cent Positive	Total Nerve Cases with per Cent. Positive
1	Wassermann.....	(1,397) 50	(723) 60	(405) 25
2	Alcoholic leproma extract....	(210) 52	(109) 75	(91) 25
3	Aqueous leproma extract....	(308) 66	(144) 84	(137) 41
4	Tuberculin.....	(180) 68	(44) 84	(17) 35
5	Bacillary emulsions.....	(89) 83	(55) 93	(12) 58

The serum in leprosy contains fixation bodies that can be recognized in about half the cases by the use of lipoidal antigens. When extracts or emulsions of acid-fast organisms are used as antigen, positive fixation can be obtained in a considerably larger percentage. With all antigens there is a striking difference between the number of cases of leprosy with skin lesions that give a positive fixation when compared with the cases having nerve lesions alone. The relatively smaller number of the latter cases giving positive reactions may be due to the absence of fixation substances in many of these cases, but possibly is an evidence of a lower concentration of such substances in the serum.

The most fundamental and serious criticism to which all the work is open, is with regard to the apparent lack of specificity of such fixation tests. With lipoidal antigens such as are used in the Wassermann reaction, the fixation test in leprosy is certainly no more specific than in syphilis. Alcoholic and aqueous extracts of leprosy tissues belong

to the same group and are open to the same objection since apparently aqueous extracts of tumors have equally good antigenic qualities. Probably the chief interest centers in the use of bacterial antigens since if specificity can be shown in the reaction, these antigens should offer a better opportunity for its demonstration than any others now available. The absence of suitable controls of tests in other diseases in the work with tuberculin as antigen renders this material of little value in the present discussion. Regarding the bacillary emulsion antigens, however, it has been noted that, together with tubercle bacilli and organisms isolated from lepers, other acid-fast bacilli act almost equally as well as antigen; that emulsions of typhoid bacilli have antigenic properties with some cases of leprosy; and that many cases of syphilis react with the bacillary emulsions as antigen. All these factors, then, throw doubt on the specificity of the fixation reactions in leprosy.

In the experimental work in the following portion of this paper, a study has been made of the fixation substances in leper serums with various acid-fast antigens by a method resembling that employed with immune rabbit serums. The specificity of the reaction has been investigated by making quantitative determinations of such antibodies with acid-fast antigens, with antigens made from nonacid-fast organisms, and with a lipoidal (Wassermann) antigen.

In the previous paper a quantitative study was made of the fixation antibodies in rabbits immunized with various members of the acid-fast group of bacteria as antigens. It was shown that in such immune serums there is a fixation antibody which reacts with many acid-fast bacteria, and that there is a tendency for certain organisms to form subgroups. This reaction is specific for this group, and may be termed acid-fast fixation. It was noted also that rabbit serums of high titer contained bodies which, in lower dilutions only, gave a nonspecific fixation with antigens made from various nonacid-fast bacteria. This nonspecific fixation is demonstrable in other serums of high titer, and may be a variety of sensitization to all bacterial protein, or associated with the lipoidal material of bacterial cells.

The present paper consists of a study of the fixation properties of a series of leper serums with acid-fast bacterial antigens. Although it may not be generally conceded that the specific cause of leprosy has been grown artificially, the disease is certainly an infection by an acid-fast organisms, and the serum might be expected to contain fixing

substances which would react with antigens of acid-fast bacteria — an acid-fast fixation.

The difficulty in securing a large series of leprosy cases in the United States unless one is situated near one of the endemic foci, is obvious. However, the chief purpose in making this study was to determine the presence in leper serums of fixation antibodies for acid-fast antigens, and to compare the concentration of such antibodies in cases of different clinical types and severity. It has been possible to do this satisfactorily with the relatively small series of cases at my disposal.

METHODS

The material for study was obtained from 15 lepers from the Louisiana Leper Home through the cooperation of Dr. Ralph Hopkins of New Orleans, and from 5 cases from the University of California Dispensary through the kindness of Dr. Howard Morrow of San Francisco. The blood was obtained in the usual way by venous puncture, and the serum sealed in ampules, inactivated and kept in the icebox until used. Some of the serums were tested immediately, but others were kept for several months before the reactions were done. In none did any anticomplementary action develop. Each serum was reactivated for 5 minutes immediately before use in the tests. This lack of anticomplementary action was attributed to the fact that the serums were sterile, sealed and kept at icebox temperature. That such serums may be kept for considerable periods with little deterioration is indicated by the fact that two serums reacting positively were retested after having been kept for 3 years with practically identical results.

The technic of the fixation test has not varied from that described previously in connection with the second series of experiments with immune rabbit serums. The antigens used were the same as those employed in the tests of immune rabbit serums reported earlier in another paper, and the amounts used in each test were identical with those used in the same tests. Unfortunately, the amount of serum available did not permit the testing of each serum with every antigen, so that certain antigens were necessarily omitted from the tests for lack of material. Every serum, however, was tested with several antigens. A simple lipoid antigen was also used in testing each of the serums. This was so-called simple alcoholic antigen such as is used in the Wassermann reaction, and was made by extraction of beef heart with ethyl alcohol in the usual way. The dose used was less than half the anticomplementary amount, and contained at least five antigenic units when tested with syphilitic serum. All patients' serums were titrated with a constant amount of antigen in making the tests. Starting with 0.2 cc of serum, gradually decreasing amounts were used until the minimal fixing dose was determined. Controls for anticomplementary action of double the antigen dose, and of the largest amount of serum used, were always included.

In table 7 is shown the minimal fixing dose of each of the various leprosy serums tested with several acid-fast antigens, and the lipoidal antigen. Some of the cases were tested also with similar antigens of

B. typhosus and a diphtheroid bacillus. The cases, a brief clinical summary of which will be found at the end of the paper, were numbered after the tests were performed in order to simplify the table. No tests of normal or control serums are shown, although many normal individuals and persons suffering from other diseases have been examined. These will be given in the following paper. It is sufficient to state here that other serums do not react with these acid-fast antigens except those from patients with tuberculosis.

TABLE 7
COMPLEMENT FIXATION WITH LEPROSY SERUMS AND VARIOUS ANTIGENS

Antigens	Minimal Fixing Dose of Serums from Leprosy Cases in c c																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>B. leprae</i> — Duval (chrome).....	0.002	0.008	0.01	0.05	0.03	0.1	0	...	0	0	0	...	0.2		
<i>B. leprae</i> — Levy.....	0.004	0.03	0.03	0.03	0	...	0	0	0.2			
<i>B. leprae</i> — B.....	0.001	0.004	0.006	0.1	0.03	0.05	0.05	0.2	...	0	0	0.2	...	0	0	...	0	0
<i>B. leprae</i> — F.....	0.001	0.006	0.03	0.05	0.1	0.1	0.1	0	0.1	0.1	0.2	0.1	0	...	0		
<i>B. leprae</i> — G.....	0.001	0.006	0.008	0.01	0.05	0.1	0	0.1	0	0	...	0	...	0.2		
<i>B. leprae</i> — H.....	0.001	0.006	0.03	0.05	0.1	0.1		
<i>B. leprae</i> — Hansen (from skin).....	0.001	0.006	0.006	0.05	0.1	0.05	0.03	0.2	0.2	0.1	0.2	0	0.2	0	0	0.2	0	0.2	0	
<i>B. leprae</i> — Kedrowsky.....	0.002	0.004	0.008	0.006	0.01	0.03	0.05	0.05	0.03	0.03	0.1	0.2	0.1	0.1	0.1	...	0.2	0.2	0.2	
<i>B. leprae</i> — Duval (nonchrome).....	0.002	0.004	0.006	0.006	0.03	0.05	0.1	...	0.1	...	0.1	0.1			
<i>B. tuberculosis</i> — human.....	0.0005	0.002	0.004	0.005	0.008	0.03	0.03	0.03	0.03	0.03	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2
<i>B. tuberculosis</i> — bovine.....	0.001	0.004	0.004	0.005	0.05	0.03	0	0.1	0.2	0.1	...	0.1	0.2	0.2	0.2		
<i>B. tuberculosis</i> — avian.....	0.001	0.002	0.006	0.006	0.01	0.05	0	0.2		
<i>B. tuberculosis</i> — turtle.....	0.001	0.004	0.05	0.01	0.03	0.05	0.2	0.2	0	0.2		
<i>B. smegmatis</i>	0.0005	0.004	0.004	0.006	0.03	0.03	0.03	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2		
<i>B. of butter</i>	0.001	0.006	0.008	0.01	0.03	0.1	0	...	0	0.2	...	0	0.2			
<i>B. of timothy</i>	0.001	0.006	0.006	0	0.05	0	0	0	0.2	0.2	0		
<i>B. typhosus</i>	0	0	0	0	0	0	0	0	0	0	0	0		
<i>B. diphtheroid</i>	0.05	0	0.1	0	0	0	0	0	0	0	0	0		
Lipoid (Wassermann).....	0.03	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0.03	0	0	0	0	0

0 means no fixation with 0.2 c c.

An examination of the results of the tests with the leper serums and the various antigens reveals several notable features that may be briefly summarized as follows: All serums give positive fixation with one or more of the acid-fast antigens, although there is a marked difference in the concentration of the fixation bodies in the different cases. In about half the serums, for example, fixation is obtained with less than 0.1 c c — one having a titer of 0.0005 c c — while the remainder required 0.1 or 0.2 c c to give a positive result. The serums of higher titer give the acid-fast group reaction with all the acid-fast antigens; but those of low titer fail to give fixation with certain of

the organisms. Of the antigens, certain ones fix with every serum tested, and have a tendency to give fixation with somewhat smaller amounts of serum than the others. Those organisms that possess a higher antigenic value with leper serums are the same strains that show a similar superiority with rabbit serums immunized against acid-fast bacteria. About half the serums were tested with antigens from two nonacid-fast organisms, in order to determine the possible presence of nonspecific fixation. Two of the serums tested, both of which belong to the group having a higher fixing titer with the acid-fast antigens, gave fixation also with one of the nonacid-fast organisms. All other serums tested were negative. With the simple alcoholic Wassermann antigen, three serums were positive; two of these have the highest titer with acid-fast antigens of any of the group, while the third is of relatively low titer.

In general, the leper serums closely resemble the serums of rabbits immunized with acid-fast organisms in their behavior with bacterial antigens in the fixation test, and this is more especially true of those leper serums that have a high titer. Although there is some difference in the titer of each serum with the different antigens, leper serums tend to fix more uniformly with all acid-fast antigens than do the rabbit serums. In other words, the group reaction or acid-fast fixation is even more evident with serum from lepers than in immune rabbit serums. This illustrates the futility of attributing any etiologic significance to an acid-fast organism grown from a leper merely because that particular organism gives a positive fixation with leper serum. Such inferences made by certain workers have obviously been due to a lack of proper control experiments with other acid-fast antigens. Additional evidence of this is given by the fact that an antigen of acid-fast bacilli obtained from the skin lesions of a case of leprosy reacts less well with leper serums than some of the saprophytic acid-fast organisms (e. g., *B. smegmatis*).

The nonspecific fixation that occurred in two of the serums with a nonacid-fast antigen resembles the similar nonspecific reactions found with immune rabbit serums of high titer and is further evidence that such a phenomenon may be common to many serums having a high antibody content. In leper serums such fixation is given only with many times the minimal fixing dose of serum for acid-fast antigens and only with serums of high titer. By the titration of a serum, therefore, nonspecific reactions are readily recognized. The chief

feature of interest in such reaction is that they may occur with certain naturally immunized human serums, as well as with artificially immunized rabbit serums.

TABLE 8
COMPLEMENT FIXATION WITH LEPER SERUMS AND HUMAN TUBERCLE BACILLI ANTIGEN

Cases	Minimal Fixing Dose of Serum (c c)	Type	Remarks
1	0.0005	Skin	Terminal stage of extensive general involvement; died a few months later
2	0.002	Skin	Actively progressing lesions
3	0.004	Mixed	Long duration; arrested
4	0.006	Skin	Active extending lesions
5	0.008	Skin	Long duration; stationary
6	0.03	Skin	Well advanced; progressing
7	0.03	Nerve	Active lesions; deformities; trophic ulcers
8	0.03	Skin	Several years duration; inactive
9	0.03	Skin	Short duration; improving
10	0.03	Skin	Long duration; improving
11	0.1	Skin	Advanced stage; improving
12	0.1	Nerve	Advanced stage; arrested
13	0.1	Nerve	Long duration; arrested
14	0.1	Mixed	Acute exacerbation; lepra fever
15	0.1	Nerve	Boy in incipient stage
16	0.2	Mixed	Improving; clinically well few months later; discharged
17	0.2	Nerve	Long duration; arrested
18	0.2	Nerve	Long duration; arrested
19	0.2	Nerve	Active anesthetic type
20	0.2	Nerve	Early anesthetic type

The relationship of the type of disease to the concentration of fixation substances in leper serum is graphically shown in table 8. In this table the minimal fixing dose of serum with human tubercle bacilli antigen only is given, since this antigen is one of those which reacted best with all serums. It is evident that the serums with the highest titer are from lepers with the nodular type of the disease, and that the cases in which the nerves alone are affected tend to have less antibody in the serum. The skin lesions of the nodular variety contain enormous numbers of acid-fast bacilli, while the purely anesthetic cases show as a rule relatively few organisms. It is of interest that the amount of fixation substances in the serum corresponds in general with the number of acid-fast organisms in the lesions.

In discussing the reaction of leper serums with lipoidal antigen, I realize that the series studied is small and that the percentage of positive reactions obtained is less than the percentage of Wassermann reactions reported by most other observers in lepers. One significant feature, however, deserves mention. The two serums of the highest fixation titer with acid-fast reactions, gave positive reactions with the

lipoidal antigen. The resemblance of this lipoidal reaction in serums of high titer and the nonspecific fixation in such serums with other antigens seems more than a coincidence. A certain number of positive Wassermann reactions found in lepers are presumably due to a coincident syphilitic infection. By control studies on nonlepers two authors (Fletcher, Sordelli and Fischer) find about 10 per cent., while the only other recorded observation (Baermann and Netter) shows 25 per cent. in individuals (Chinese) showing no signs of leprosy. It is likely that a titration of serum with both lipoidal and acid-fast antigens would show a higher titer with the former in syphilitic lepers. Such a difference in titer was noted in the third positive case (case 10) observed in this series and suggests that in this instance a syphilitic infection may be the cause of the positive reaction rather than leprosy. As mentioned earlier, however, the proportion of positive Wassermann reactions reported in lepers is higher than can be explained by coincident syphilis, and must depend on some additional factor. The present experiments indicate that a positive fixation with leper serums and lipoidal antigen is obtained with those cases having a high concentration of fixation antibodies with acid-fast organisms, and that this is related to the nonspecific fixation found in serums of high titer with a number of antigens. It seems probable, therefore, that, in addition to the positive Wassermann reaction occurring in syphilitic lepers, an additional number of lepers whose serum contains a high concentration of leprous antibody give a nonspecific reaction with lipoidal antigen. It is significant in this connection that in the reports from the literature (table 1) a very much higher percentage of positive Wassermann reactions has been noted in cases of nodular leprosy, and it is in this type that the concentration of fixing bodies is greatest.

That certain serums have a rather high concentration of fixation antibodies, and that these serums react best with a number of antigens, may have some relation to the reports of fixation with leprosy serum and various antigens that have been reviewed in the early part of this paper. Since practically all of the observers used 0.1 c c of serum in the tests, it is quite probable that the leper serums that reacted positively were those with higher concentrations of complement-fixing antibodies. It will be remembered that as the observations were grouped on the basis of the type of antigen used, an increasing percentage of positive tests accompanied the use of antigens approaching

acid-fast bacillary emulsions, the highest percentage being given with the latter type of antigen. This may be explained by assuming that a gradually increasing number of the serums having a lower concentration of antibodies react with such acid-fast antigens or, in other words, give a specific acid-fast fixation. The fact that every serum of the group studied in these experiments gave a positive acid-fast reaction was unexpected, and probably due in part to the somewhat more delicate methods of fixation used. The additional advantage of having several antigens of the group tested that have uniformly been capable of giving fixation with small amounts of serum, may also have been a factor. Since the concentration of fixing bodies is so low in some of the serums studied as to require 0.2 c c for a positive test, it is quite probable that if the series had been amplified certain serums would have been found containing still fewer of these antibodies, so that even 0.2 c c of serum would contain an insufficient amount to give a positive reaction. There is considerable evidence to indicate that this variation in antibody content of different leper serums is one of the important factors on which depends the difference in the number of positive results reported by various observers and is probably quite independent of the type of antigen used.

SUMMARY

Leper serums contain complement-binding substances that react with antigens of acid-fast bacilli and give an acid-fast fixation similar to that obtained with serums of rabbits immunized with acid-fast organisms. Some serums contain these antibodies in rather high concentration, notably those from cases with the nodular type of the disease; other serums show a relatively low antibody content. The serums of high titer may give a nonspecific fixation also with non-acid-fast antigens and with lipoidal (Wassermann) antigen, but only in comparatively low dilutions. It is suggested that this attribute of such high titer leper serums may explain a certain percentage of positive Wassermann reactions described in leprosy. Among the acid-fast antigens studied, certain organisms have superior antigenic properties in complement-fixation tests with leper serums. The acid-fast reaction given by leper serums with acid-fast bacterial antigens prevents the use of the complement-fixation reaction in obtaining evidence of the etiologic importance of any acid-fast organism isolated from leprosy.

BRIEF DESCRIPTION OF LEPROSY CASES

1.—Nodular type; Louisiana Leper Home. Old colored man with general infection of skin most marked on face and hands, little nerve involvement. Disease was in terminal stage and progressed rapidly to fatal outcome a few months later.

2.—Nodular type; University of California Dispensary. An Armenian, 40 years old, with actively progressing tubercular skin lesions of unknown duration.

3.—Mixed type; Louisiana Leper Home. Middle-aged white man with leprosy of long duration but not virulent in type. Evidences of the disease in both nerves and skin, but little progression in either.

4.—Nodular type; University of California Dispensary. White boy, 19 years; with actively extending erythematous lesions generally distributed. Duration, 8 months.

5.—Nodular type; Louisiana Leper Home. An old man with skin lesions of long duration. Disease apparently stationary.

6.—Nodular type; University of California Dispensary. A young Chinese with extensive skin lesions of several months' duration.

7.—Nerve type; Louisiana Leper Home. A young white man with active nerve lesions; trophic ulcers, deformity and mutilation.

8.—Macular type; Louisiana Leper Home. A young white man with macular lesions of several years' duration. The disease has not advanced beyond the incipient stage and is improving.

9.—Macular type; Louisiana Leper Home. An old white man with macular skin lesions of short duration; disease is in incipient stage and improving.

10.—Nodular type; Louisiana Leper Home. Young white man with advanced skin lesions. Condition is improving.

11.—Macular type; Louisiana Leper Home. Incipient case with macular lesions of skin. Disease is improving.

12.—Nerve type; Louisiana Leper Home. A middle-aged white man with nerve lesions of more than 15 years' duration. Disease apparently arrested.

13.—Nerve type; Louisiana Leper Home. A middle-aged white man with trophic changes of long duration. The disease is still somewhat active.

14.—Mixed type; Louisiana Leper Home. A middle-aged white woman with both skin and nerve involvement of one year's duration. When the blood was taken the disease was virulently progressive and in a stage of acute exacerbation (lepra fever) that lasted 5 months. On subsidence of fever and inflammatory reaction of lesions, there was considerable improvement.

15.—Nerve type; University of California Dispensary. A boy, 15 years old, with lesion involving right ulnar nerve and its distribution in arm and hand.

16.—Mixed type; Louisiana Leper Home. Middle-aged white man in incipient stage with anesthesia of ulnar nerve distribution and infiltrated patches on skin of face, neck and arms. Patient was improving when specimen was taken and in few months all clinical and microscopic evidence of the disease had disappeared and patient was discharged from the Home.

17.—Nerve type; Louisiana Leper Home. A middle-aged white man with trophic lesions of more than 30 years' duration. The disease is apparently arrested after considerable mutilation and deformity of the extremities.

18.—Nerve type; Louisiana Leper Home. Same class as case 17.

19.—Nerve type; Louisiana Leper Home. Middle-aged colored man. Active anesthetic type with evidences of active degeneration of nerves.

20.—Nerve type; University of California Dispensary. A Japanese man, 32 years old, with diffuse infiltrating plaque of 8 months' duration covering left cheek and temple with anesthesia of lesion and surrounding area.

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COMPLEMENT FIXATION WITH ACID-FAST BACTERIA

III. IN TUBERCULOSIS

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Complement fixation with serum from tuberculosis and antigens of tubercle bacilli or their products, has been the subject of a number of investigations. No other bacterial antigen has been so widely used in testing human serum. The earlier work, after the first use of the test by Widal and Le Sourd¹ in typhoid fever and Bordet and Gengou² in tuberculosis, was somewhat contradictory and was received with much skepticism because of the lack of conformity between the clinical cases and the fixation tests. It is believed that these earlier discrepancies may have been due to the imperfect knowledge of some of the technical difficulties of the fixation reaction and also to the nature of the antigens used. Within the past few years, however, increasing numbers of reports have appeared in which the results of the tests have corresponded closely to the clinical analyses of the cases so that confidence in the diagnostic value of the test in tuberculosis is increasing. Many who have studied the reaction feel that its value when properly done is quite comparable to that of the Wassermann reaction in syphilis.

A review of the work already reported, which comprises more than one hundred articles, will not be given here since a summary of the more important papers may be found in recent publications of Craig,³ Miller,⁴ Moon,⁵ Bronfenbrenner,⁶ Stoll and Neumann,⁷ and others. It may be stated that the antigens that have proved most satisfactory are culture filtrates and emulsions of the tubercle bacillus. A high percentage of positive reactions in known clinical tuberculosis has been the rule with the use of these antigens. One discouraging feature in the use of the test is a nonspecific fixation that has been noted in cases not clinically tuberculosis, particularly in syphilitics with strongly positive Wassermann reactions. The number of syphilitics giving such positive reactions has varied, but has frequently been from 20 to 40%. This has led to a number of modifications in the antigen in order to secure preparations that would not react with serums from syphilitics and yet give fixation with a large percentage of cases of tuberculosis. The more recent reports

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¹ Compt. rend. Soc. d. biol., 1901, 53, p. 841.

² Compt. rend. Acad. d. sc., 1903, 137, p. 351.

³ Am. Jour. Med. Sc., 1915, 150, p. 781; Jour. Am. Med. Assn., 1917, 68, p. 773.

⁴ Jour. Am. Med. Assn., 1916, 67, p. 519; Jour. Lab. and Clin. Med., 1916, 1, p. 816.

⁵ Jour. Am. Med. Assn., 1918, 71, p. 1127.

⁶ Arch. Int. Med., 1916, 17, p. 492.

⁷ Jour. Am. Med. Assn., 1919, 72, p. 1043.

indicate that several of the antigens in use are capable of giving from 85 to 95% of positive reactions with cases of clinical tuberculosis, while less than 10% of syphilitics react positively, or no more than can be assumed to have a coincident tuberculosis. No antigen has yet been described, however, that can be considered an entirely satisfactory preparation and none gives uniform results in the hands of every investigator. Possibly this is due in part to a lack of standardization of the technical methods used in performing the test, and probably also to the lack of keeping qualities of some of the antigens used.

It is this inability on the part of several workers to verify the more favorable results with the test even when using the same or similar antigens that has prevented a more widespread confidence in its value. The explanation of the disparity in results in these cases is not clear. One point, however, may be mentioned regarding the instability of bacterial antigens. Only those who have had a considerable personal experience with antigens of this type realize the exacting care necessary to preserve their efficiency. Bacterial contamination, for example, not only leads to the appearance of anticomplementary substances, but such contaminated antigens may also give nonspecific fixation, or "false positives." Deterioration in fixing quality of an antigen frequently occurs after a time, and must be detected immediately. Constant and careful control of a bacterial antigen, therefore, is necessary to obtain comparable and reliable results.

Some investigators have attempted to determine the relation of the complement-fixation test to the clinical activity of the disease. The results show that while the clinically active cases give a very large proportion of positive reactions, the inactive or arrested cases vary from 9% (Miller²) to 61% (Brown and Petroff³). The value of such studies is considerably lessened by the difficulty in classification of lesions by clinical examination as active or inactive. Although it is possible to demonstrate definite clinical signs indicating an active pathologic process in one group, the other group of "inactive," "arrested" or "latent" tuberculosis may be expected to contain cases with active lesions not evidenced by clinical signs of activity. It is possible that a positive complement-fixation test may indicate an active or potentially active pathologic lesion but confirmation of such a belief must depend on other factors than clinical activity.

This paper deals with complement-fixation tests with serums from patients with tuberculosis using a number of acid-fast bacterial antigens, together with similar tests in syphilis and a variety of other diseases. As in the preceding experiments with immune rabbit serums and leper serums, the concentration of the complement-fixing antibodies has been studied by titration.

METHODS

The antigens used have been identical with those employed in the study of leper serums and the technic of performing the tests also has not varied from that described previously in connection with the same experiments. Since the titration of the serums was made by using decreasing amounts from 0.2 cc, an additional control or natural hemolysin was added for each serum. This consisted of 0.1 cc of serum with 2 units of complement, incubated with the other tests. At the end of 45 minutes, unsensitized sheep cells were added to this tube. Fifteen minutes later, when the tests were removed from the water

² *Am. Rev. Tuberc.*, 1918, 2, p. 525.

bath, this control tube showed complete hemolysis in about one third of the serums tested. In such cases unsensitized cells only were added to those tubes containing 0.1 and 0.2 cc of that serum, since sufficient natural hemolysin was present for the hemolytic system. Cells sensitized with 2 units of hemolysin were added to all tubes containing less than 0.1 cc of serum and in all cases where 0.1 cc of serum did not contain enough natural sensitizer to luke the cells completely in 15 minutes. By this method the danger of any large excess of hemolysin was minimized. The absorption of natural hemolysin from all serums before testing them is somewhat tedious and does not seem necessary.

The serums tested were from several sources, most of them from patients in the University of California Hospital, and the San Francisco Hospital. The syphilitic serums, all of which gave strongly positive Wassermann reactions, were obtained through the kindness of Miss Miriam Olmstead of the Presbyterian Hospital and Dr. R. Ottenberg of the Department of Bacteriology, Columbia University, New York. Some of the serums from tuberculous cases were furnished by Dr. H. R. Miller of the latter laboratory. All serums were inactivated at 56 C. for 30 minutes and were tested within 1 week of collection from patients. The necessary controls were always included.

TABLE 1 .
COMPLEMENT FIXATION WITH VARIOUS ACID-FAST ANTIGENS AND SERUMS FROM TUBERCULOSIS

Antigens	Minimal Fixing Amount of Serum in c c						
	Gener- alized Tubercu- losis in Monkey	Pul- monary Tuber- culosis, Baecilli in Sputum	Tuber- culous Glands of Neck	Tuber- culous Epi- didym- itis	Pul- monary Tuber- culosis, Baecilli in Sputum	Tuber- culous Spon- dyilitis	Pul- monary Tuber- culosis, Baecilli in Sputum
B. leprae—Duval (chrome)....	0.006	0.006	0.008	0
B. leprae—Levy.....	0.008	0.006	0.01	0
B. leprae—B.....	0.008	0.004	0.006	0	0.004	0
B. leprae—F.....	0.006	0.006	0.05	0
B. leprae—G.....	0.006	0.008	0.03	0
B. leprae—H.....	0.006	0.01	0.05	0
B. leprae—Kedrowsky.....	0.006	0.004	0.01	0.05	0.004	0.004	0.2
B. leprae—Duval (nonechrome)	0.006	0.006	0.01	0.2
B. leprae—Hansen (from skin)	0.008	0.004	0.008	0
B. tuberculosis—human.....	0.004	0.004	0.008	0.03	0.004	0.004	0.2
B. tuberculosis—bovine.....	0.004	0.006	0.05	0.006	0
B. smegmatis.....	0.004	0.004	0.03	0.1	0.004	0.004	0.2
B. of butter.....	0.008	0.006	0.03	0.006	0
B. diphtheroid.....	0	0	0	0	0	0	0
Lipoid.....	0	0	0	0	0	0	0

0 means no fixation with 0.2 c c.

The results of the present series of tests have been arranged in 3 tables. Table 1 gives examples of the fixation test in tuberculous serums with a variety of acid-fast antigens; table 2 shows the reactions obtained with a number of cases of tuberculosis and a variety of other conditions, including syphilis; while table 3 illustrates the concentration of antibodies in syphilitic cases with a simple lipoidal antigen. In all instances the concentration of the fixing substances in the serums is given.

Although a considerable number of serums from cases of tuberculosis have been tested with several of the acid-fast antigens; the results have been so uniform that it seems unnecessary to show in detail more than a few typical examples. In table 1, tests of seven serums

with different antigens are given. All these showed active tuberculosis, 3 with pulmonary involvement, 3 in which bone, lymph node, and epididymis, respectively, were affected, and 1 of generalized tuberculosis in a monkey. The length of time required for titrating every serum with a number of antigens prevented the use of this procedure in a large series, but enough serums were tested to show that in tuberculosis, just as in leprosy and in rabbits immunized with acid-fast organisms, the serum contains substances that give fixation with members of the acid-fast group of bacteria. The reaction is not specific for the tubercle bacillus, but is an acid-fast fixation specific for the group of organisms. In a large percentage of the cases tested several antigens were tried, including a pigmented and a nonpigmented leprosy strain, a purely saprophytic organism like the smegma bacillus, and the tubercle bacillus. The variation of the different acid-fast antigens in their ability to give fixation with tuberculosis serums is slight, although, as with other serums, some strains are more constantly capable of fixing with smaller amounts of serum than others. Among the best of the antigens tested is *B. tuberculosis* (human). None of the serums studied showed a nonspecific fixation with an antigen prepared from a nonacid-fast diphtheroid bacillus. The difference in titer of the fixing bodies in different cases is striking and seems to bear no relation to the clinical type or severity of the disease.

Since the tubercle bacillus in all tests appeared to be one of the best of the acid-fast antigens, the results of its use in a number of cases are shown in table 2. In the 91 cases of tuberculosis, 90% gave positive fixation, 2 of 6 cases of suspected tuberculosis were positive, while 5 cases of healed pulmonary lesions were all negative. In a certain number of instances in this series, cases of "suspected tuberculosis," when found to have a positive acid-fast fixation, were subject to a more thorough clinical study which resulted in the demonstration of tubercle bacilli in the sputum. Fifty cases of acquired syphilis, all of which gave strongly positive Wassermann tests, showed 5 positive acid-fast fixations, a proportion slightly larger than the 5 positive cases of a group of 75 tests on persons with other diseases. This last group included cases of such acute infections as typhoid, scarlet and rheumatic fevers, pneumonia, diphtheria, endocarditis, pyogenic osteomyelitis, and malaria, as well as chronic processes like carcinoma, bronchial asthma, cardiorenal disease, various lesions of the central nervous system, chronic bronchitis and emphysema. The 5 positive reactions were given by patients with acute pulmonary infection

(influenza?) inoperable cancer of the stomach, chronic nephritis, polyserositis and by a healthy nurse on duty in a tuberculosis ward.

Among the tests is included a group of serums from various diseases received from a clinician who saw many cases of tuberculosis and was interested in the test, but somewhat skeptical about its value. These serums were marked by numbers only and the clinical diagnosis was not given until after the results of the tests had been reported. The high percentage of positive reactions given by cases of clinical tuberculosis convinced this physician that the test is of definite clinical value.

TABLE 2
COMPLEMENT FIXATION IN TUBERCULOSIS AND OTHER DISEASES WITH TUBERCLE BACILLI ANTIGEN, SHOWING CONCENTRATION OF FIXING BODIES IN THE SERUMS

Diagnosis	Total Number Tested	Number Negative	Minimal Fixing Dose of Serums in c c							
			0.2	0.1	0.05	0.03	0.01	0.008	0.004	0.002
Tuberculosis—pulmonary.....	78	8	4	17	18	14	6	5	5	1
Tuberculosis—lymph glands.....	6	1	2	1	1	1
Tuberculosis—bones.....	6	0	..	3	2	1	..
Tuberculosis—epididymis.....	1	0	1
Tuberculosis—suspected.....	6	4	..	1	1
Tuberculosis—healed.....	5	5
Syphilis—Wassermann positive.....	50	45	2	2	1
Various other diseases.....	75	70	1	3	1

In all serums the smallest amount giving fixation with the antigen has been determined by titration, and the results (table 2) illustrate the relatively wide variation that may occur in the concentration of fixing antibodies in different serums. No relation could be shown between the titer of a serum and the clinical severity of the infection. It is very significant that such variation in titer does occur and in this may lie the explanation of the fact that certain cases of undoubted tuberculosis give negative reactions. In such instances, the concentration of antibodies is so slight that their recognition is not possible by the complement-fixation method, or the fixing substances may be entirely absent. When, for example, it is possible to obtain fixation with as little as 0.002 c c of serum in some cases with an acid-fast antigen, the reaction appears delicate and the antigen reliable. When other cases show fixation with only 0.02 c c or 0.2 c c, one is justified in saying that the reaction is as delicate and the antigen as reliable, but that less fixing bodies are present in such serums. A small group of cases in which too small an amount of fixing antibody is present for fixation in 0.2 c c must be considered an example of the limitation of the method. The discovery of an antigen preparation that will give positive reactions with every case is improbable.

A similar titration of the serums from 50 cases of syphilis with a simple lipid antigen is given in table 3. One object of such a study was to determine whether any relation existed between the presence of a high concentration of syphilitic antibody in certain serums and a positive acid-fast fixation. No definite relationship was found. Of the 5 serums that gave positive acid-fast fixations, the titer with lipid antigen was 0.004 c c in 3, while in the others it was 0.006 c c and 0.008 c c. Only strongly positive syphilitic serums were tested. An analogy between the fixation reaction of syphilitic serums with lipid antigen and acid-fast fixation in serums from tuberculosis may be pointed out by comparison of the titration data given. In both instances, the concentration of complement-fixing antibodies varies

TABLE 3
COMPLEMENT FIXATION IN SYPHILIS WITH SIMPLE LIPOIDAL ANTIGEN, SHOWING CONCENTRATION OF SYPHILITIC ANTIBODY IN SERUMS

Diagnosis	Number Tested	Minimal Fixing Dose of Serums in c c								
		0.05	0.03	0.01	0.008	0.006	0.004	0.002	0.001	0.0003
Syphilis—acquired.....	50	6	15	4	5	7	8	2	2	1

considerably in different cases. It has been suggested that certain cases of tuberculosis do not give a positive acid-fast fixation because of an unrecognizably small amount of antibody in the serum, and a similar lack of syphilitic antibody probably explains the negative Wassermann reactions in certain cases of syphilis. Although no such instances were recognized in this series, there seems to be no doubt that these cases are not infrequent. Warthin⁹ has found at necropsy active syphilis with spirochetes in many cases giving negative Wassermann tests during life, and the frequency of negative blood Wassermann reactions in syphilis of the central nervous system is well known. Since complement-fixing bodies are apparently subject to considerable variation in concentration in the blood serum in both syphilis and tuberculosis, it is not surprising that certain cases contain insufficient amounts to be recognized.

Although most workers with the test in tuberculosis agree that the antigen should contain a number of strains of tubercle bacilli to obtain the best results, there is some evidence from these experiments that at least one other factor is important in the preparation of a good antigen. It has been mentioned previously that certain acid-fast

⁹ Am. Jour. Med. Sc., 1916, 152, p. 508.

antigens are superior to others in fixing with smaller amounts of antibody in immune serums, and that these antigens give the most uniform suspensions in salt solution. In two instances only (*B. smegmatis* and *B. tuberculosis*) have different strains of the same organism been studied and in both cases the strain that gave the most even suspension made the better antigen. So consistently has this physical characteristic of the bacillary suspension been associated with the ability of an antigen to fix with small amounts of antibody in serum, that the property of forming a uniform, fine suspension in salt solution seems essential in the best antigens. Apparently, strains which contain a relatively large amount of waxy material do not lend themselves so well to saline suspension and form less delicate antigens. It seems quite possible that similar variations in different cultures of tubercle bacilli may be encountered.

The amount of antigen used in the test is of much importance, since large amounts of antigen tend to give nonspecific fixation irrespective of any anticomplementary action of the antigen itself. This is especially true with regard to syphilitic serums. Various bacterial cultures act as antigen in the Wassermann reaction if used in sufficient amounts and this is true also of tubercle and other acid-fast antigens. It is quite possible, however, to employ amounts of antigens which will give fixation with tuberculous serums, but which are insufficient to react with syphilitic serums. Such an adjustment of the proper antigen dose may be ascertained by preliminary titrations with several serums. As an additional safeguard, it seems advisable to include a control in each test with a simple lipid antigen.

A method by which a comparison of antigens may be made is by titration of the minimal fixing dose of one or more serums. This shows which antigen is capable of giving fixation with the least amount of antibody, and seems preferable to the testing of fixed amounts of serums from a number of cases to determine which antigen reacts with the highest percentage.

The strength or intensity of the reaction with any serum is estimated more accurately by a titration of the serum than by an attempt to estimate the amount of hemolysis in a single tube, although such a titration involves additional time. The severity of the tuberculosis is not always indicated by the strength of the fixation, but those serums with a titer of less than 0.1 c c are practically always from cases that are clinically active. The large amount of blood necessary and the increased danger of anticomplementary action of the serum are among

the objections to using more than 0.2 c c as a maximum amount in the test. It is probable that some additional cases might give positive reactions with larger amounts of serum. Only relatively fresh serums or those that have been kept sterile and sealed should be used for the test. Serums that give negative reactions may become positive after bacterial contamination, and this change is associated with the development of anticomplementary action, since such serums if tested later are anticomplementary. Positive reactions obtained on old serums or those that have developed even slight anticomplementary properties are therefore unreliable. Complement-fixing substances do not disappear for many months, however, if serums are kept sterile.

The type of antigen most suitable for complement-fixation tests in tuberculosis is still somewhat in dispute. That used in these experiments has proved satisfactory, but no comparative study has been made with other antigens made from whole bacillary emulsions. Partial antigens and filtrates from cultures are open to certain theoretical objections since they do not contain all the antigenic substance. After extraction of bacilli with fat solvent, both the residue and the extract will act as antigen, and similarly both the filtrate (tuberculin) and the bacillary bodies from a broth culture have been used as antigens. In order to have all the antigenic substance possible it would seem of advantage to use whole cultures. The keeping qualities of dried antigens and the ease with which a standard suspension can be made, are distinct advantages.

SUMMARY

In tuberculosis, the serum contains complement-binding substances that give fixation when members of the acid-fast group of bacteria are used as antigen. These antibodies show a considerable difference in concentration in different serums, but this difference in titer does not correspond with the severity of the infection in the different cases. A relatively small percentage of patients with active tuberculosis have too small an amount of complement-fixing antibody to be recognized in the test. These cases are analogous to those of syphilis that give a negative Wassermann reaction. The test is of some value in calling attention to unrecognized tuberculosis, but does not always indicate a clinically active process.

Leprosy and tuberculosis are the only human infections that give the reaction, and since it is specific for the acid-fast group of bacteria, the term acid-fast fixation seems appropriate.

AMEBIC DYSENTERY IN CALIFORNIA *

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AND

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The conditions of the war have greatly increased the amount of attention given to the intestinal protozoa of man, of which *Endamoeba dysenteriae*, *Giardia intestinalis*, *Trichomonas intestinalis*, and *Balan-tidium coli* are the most important. During the past 3 years, army medical journals and the journals of the schools of tropical medicine have devoted much space to the publication of the results of investigations of the problems of incidence, spread (especially by cyst carriers), diagnosis, and treatment of the respective diseases caused by these organisms. One of the most significant outgrowths of this work is the decided addition to the already accumulated evidence that amebiasis is by no means restricted to the tropics, but can become and is becoming constantly more widely established in countries of the temperate zones. The problem of the danger of the spread of amebiasis and giardiasis by returning troops is receiving especial consideration in England, and English physicians have found that many returning soldiers had acute dysentery, or were discharging the cysts of *E. dysenteriae*. A special study of 20 "cyst carriers" was made by members of the staff of the Liverpool School of Tropical Medicine.¹ Of the twenty, 14 had contracted the disease in France, and 10 of these in the region of the Somme. Two had had the disease before, one in South Africa and one in Gallipoli. Probably the first diagnosis of amebic dysentery in the United States was made in 1890 (Osler²). Since then an increasing number of cases have been reported each year in medical literature. Ten years ago Patterson³ reported amebic dysentery from 24 different states and the District of Columbia. Since that time many states have been added to this number until now nearly

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¹ Carter, H. F.; Mackinnon, D. L.; Matthews, J. R., and Smith, A. M.; *Ann. Trop. Med. & Parasit.*, 1917, 11, p. 27; Yorke, W.; Carter, H. F.; Mackinnon, D. L.; Matthews, J. R., and Smith, A. M.: *ibid.*, p. 87.

² *Med. News*, 1902, 15, p. 673.

³ *Am. Jour. Med. Sc.*, 1909, 138, p. 198.

every one of the 48 is included. Many of the states reported by Patterson were among the most northern, as Maine, New York, Ohio, Minnesota and Montana. The work of Smithies⁴ in making 1,000 routine examinations for intestinal protozoa gives an insight into the conditions in the United States at the present time regarding the incidence of intestinal protozoa. Of entameba, both histolytica and tetragena stages, there were 23 cases, while infections with *Cercomonas*, *Trichomonas*, *Giardia* (*Lamblia*), *Megastoma*, and *Balantidium* made a total of 93 persons harboring protozoa. The distribution of these cases is significant, for of the 13 different states of which these infected persons were residents, 9 are entirely north of the latitude of the Ohio river. One of these cases was from northern California.

In 1909, Long⁵ discussed the dangers to California from amebic dysentery. His attention was called to this subject by the admission, during 1907 and 1908, of 4 cases of this disease into the U. S. Marine Hospital, San Francisco. In all 4 of these cases, the disease appeared to have been contracted in San Francisco. In fact, one was a man who had never been out of the city. This led Long to institute routine fecal examinations for amebas. As a result of these examinations, between Dec., 1908, and June, 1909, he diagnosed 40 cases of amebic dysentery. Fourteen of these he believed had originated in the United States. In a paper by Gunn⁶ is the following significant sentence: "In nine of the twenty-one cases herein reported the disease was contracted in California."

In order to secure some further insight into the seriousness of the protozoan dysentery problem in California, the Division of Parasitology of the California State Board of Health sent a circular letter to 45 hospitals at widely distributed points throughout the state. In this letter inquiry was made whether cases of amebic or flagellate dysentery had been diagnosed recently. Whenever such cases were found, an attempt was made to obtain as much as possible of the case history. As a result of the circular letter, a total of 15 cases of amebic dysentery were reported from 8 hospitals. No cases of dysentery caused by flagellate protozoans were reported. On account of the absence of the attending physician in military service, the histories of 3 of these cases could not be obtained. Several additional cases were

⁴ Ibid., 1918, 156, p. 173.

⁵ California State Jour. Med., 1909, 7, p. 199.

⁶ Ibid., 1918, 16, p. 240.

reported from other sources, so that finally the desired information concerning 17 cases was secured. Table 1 summarizes such of this information as pertains to place and source of infection.

TABLE 1
SUMMARY OF CASES REPORTED TO THE DIVISION OF PARASITOLOGY

Case	Age, Years	Sex	Foreign Residence or Travel	Place and Source of Infection
1*	..	Male	California. In habit of drinking unboiled water from Sacramento river
2*	..	Male	Probably never out of California	California. Was working in a restaurant when first attack occurred
3	..	Male	In Philippine Islands in 1898	Philippine Islands
4	36	Male	Had been in Porto Rico, Cuba, and the Philippine Islands	Philippine Islands. Became ill while there in 1900
5	11	Female	Born in Honolulu.....	Hawaii. History does not indicate source of infection
6	9	Male	Born in Hawaii.....	Hawaii. "Diet gives no hint of source of infection"
7	..	Male	Had traveled in tropics.....	Probably while in tropics
8	..	Male	From Mexico.....	"No opinion"
9	..	Female	Born in Austria, lived in Japan, 9-4 years ago	History does not show
10	..	Male	In army. In Philippines 4 years	Philippine Islands. Probably drinking contaminated water or eating vegetables irrigated by such water
11*	..	Female	Never been in Orient or tropical countries. Not out of California for long time previous to infection	California. Probably from vegetables from oriental truck gardeners
12	42	Female	Residence in Hawaii about 18 years ago	Probably in Hawaii
13	..	Male	From Mexico.....	In Mexico
14*	..	Female	Never outside of California...	California. No clue as to source of infection
15*	..	Female	Resident of California all her life	California. No suggestion as to source of infection
16*	31	Male	Born in California and had never been out of state	California. First attack while living at bunk house of mining camp. Probably from food or water
17	36	Male	In Panama, 1906-1911.....	First diagnosis at Colon, Canal Zone

* Disease undoubtedly contracted within the state of California.

Of the 17 cases in table 1, there are 6 in which the disease seems to have been contracted within the state. Residence or travel in tropical countries usually makes absolute establishment of the fact of contraction of the disease within the bounds of the state questionable on account of the possibility of its having remained latent for an indefinite period following infection. When the patient has never been out of the state, however, there is no possibility of question. A definite clue to the mode of infection was hard to obtain, but one of the infections was probably caused by drinking river-water, unboiled (case 1). In another case (case 11), the disease was suspected of having resulted from eating uncooked vegetables from a truck garden

conducted by orientals. This preliminary survey combined with the findings of Long⁵ and of Gunn⁶ seems to leave no room for doubt that amebiasis has become established in California.

In all of our cases the dysenteric symptoms were sufficiently pronounced so that the patient secured the services of a physician. When the disease is in this acute stage, unencysted, active forms of the amebas are voided almost exclusively. These motile stages of *Endameba dysenteriae* are very sensitive to change and do not withstand conditions outside the body of the host. So an acute case of amebiasis would be only exceptionally responsible for the spread of the disease. In cases in which the organisms have been introduced into the alimentary tract but have not produced intestinal disturbances, the parasites pass through their normal life cycle, and when voided in the feces have completed encystment so that they are very resistant. Carriers, i. e., those who harbor *E. dysenteriae* and are voiding cysts but show no dysenteric symptoms, are probably much more common than is generally suspected. Cases of carriers do not ordinarily come to the attention of physicians, and come to light only through extensive routine fecal examinations. Such examinations of a general population are seldom made, and are made in hospitals only on some prompting suspicion. It is suggestive here to review the findings of MacAdam and Keelan⁷ among 946 British soldiers, who had served in Mesopotamia. They revealed absolutely no dysenteric symptoms, but 154 persons, or 16.2%, were found to be carriers of *E. dysenteriae*.

The condition of carrier may also arise following the treatment of acute cases, when the dysenteric symptoms disappear completely and yet the amebas remain in reduced numbers. Where routine examination follows treatment for a month or more a large percentage of "relapses" are found. The relapse may be acute or it may result in making the patient a cyst carrier.

The cases in table 1 give no clue to the problem of cyst carriers in California. The only evidence on this problem locally is that yielded by routine fecal examinations that are being made by the Division of Parasitology of the California State Board of Health of the inhabitants of the "delta region," a rich agricultural district of central California. The survey is only well started, but already a number of cyst carriers of *E. dysenteriae*, *E. coli*, *E. nana*, and *G. intestinalis* have been found,

⁷ Indian Jour. Med. Research, 1917, 5, p. 239.

showing that such carriers do exist within the state. The data so far obtained are insufficient to justify conclusions regarding frequency.

The evidence is sufficient to show that amebiasis is frequently contracted in California and that carriers occur. It is important to consider the conditions that make possible the introduction and spread of this disease into new regions. It is the cyst stage, usually found in persons showing no symptoms of the disease, that makes distribution possible. The cysts of *E. dysenteriae* are spherical or subspherical, thin walled, greenish-gray in color, and contain from 1 to 4 nuclei and often a refractile chromatoidal body. The chromatin is distributed peripherally in the nucleus with a very distinct karyosome. The cysts vary from 5-20 mikrons in size, but occur in 2 strains, one with an average diameter of 7.7 mikrons and the other with an average diameter of 12.6 mikrons (Smith⁸). Knowledge of respective sizes is quite necessary in the essential matter of distinguishing *E. dysenteriae* from *E. coli* and *E. nana*. A number of recent papers^{1, 8, 9, 10, 11, 12, 13} dealing with intestinal protozoa are valuable as an aid in making the necessary distinctions between the cysts of the various forms. The walls of the cysts of *E. dysenteriae* are very impervious and make fixation and staining difficult. However, either double gram salt solution or Donaldson's stain works very well for diagnostic purposes.

The recent work of Wenyon and O'Connor¹² shows the degree of resistance of the cysts. The cysts of *E. dysenteriae* apparently do not withstand drying, for after complete drying of the feces they stain at once with eosin. The foregoing authors use this reaction to eosin as an indication of death. In the presence of moisture, however, they continue viable for a long period. They will survive at least one month in polluted water—the less concentration of sewage, the longer the survival. Of the ordinary reagents used in water purification, Wenyon and O'Connor found that chlorinate of lime (1 part Cl in 700,000) had no action on the cysts, free chlorin in 1:10,000 concentration failed to kill in 4 hours. Carbolic acid (1:40) killed in 15 minutes, while 1:100 was lethal after 7 hours. Cresol, 1:20, killed immediately. Emetin hydrochlorid, 1:200, failed to kill the cysts in 9 hours, though a strength of 1:10,000 will kill the ameba in the active stage.

⁸ Ann. Trop. Med. & Parasitol., 1918, 12, p. 27.

⁹ Matthews, J. R.: 1918 Observation on the Cysts of the Common Intestinal Protozoa of Man, Ann. Trop. Med. & Parasitol., 1918, 12, p. 17.

¹⁰ Dobell, A., and Jepps, M. W.: Brit. Med. Jour., 1917, 1, p. 607.

¹¹ Knowles, R., and Cole, A. F.: Indian Jour. Med. Research, 1916, 4, p. 498.

¹² Kofoid, Kornhauser, Swezy, Arch. Int. Med. 1919, 24, p. 35.

¹³ Human Intestinal Protozoa in the Near East, 1917.

This brief summary of the resistance of the cysts of *E. histolytica* indicates to some extent those climatic and sanitary conditions that favor the spread of the disease. Countries with long, dry seasons, or those with long periods with temperature below the freezing point will find in natural conditions a considerable check on the distribution. However, conditions might occur in almost any country at almost any time under which there would be sufficient moisture and a high enough temperature to make possible the transfer of the cysts in a viable condition from one person to another.

The long survival of the cysts in water is one of the most important factors in the spread of amebiasis. Many streams receive untreated sewage from villages and cities, and from many such rivers, water is taken at points below for drinking purposes. When cities are using such water, bacterial examinations are usually made to determine its fitness, but these give no clue to the presence of the cysts of *Giardia* or *Endamoeba*; but when such water is used by individuals for household purposes it is unusual to make any tests whatever, and in many instances the water is drunk without boiling or treatment. Such conditions would seem to be admirable for the spread of intestinal protozoa.

Unpotable water used for irrigation purposes may also aid in distribution, for the sources of such water are given little attention. Not infrequently irrigation ditches are the most convenient means for sewage disposal. People working on tracts supplied by ditches so used are likely at times to come in contact with the water, and the introduction of the cysts into the mouth is not at all impossible.

Another way in which irrigation water may become an indirect factor is in its use in truck gardening. The use of polluted water or sewage in the irrigation of such vegetables as lettuce, celery, watercress, cabbage, etc., which are eaten uncooked, is a serious menace. Within the state are large areas devoted to truck gardens, a large part of which are in the charge of orientals or employ oriental labor. In the countries from which these people come amebic dysentery is common, and undoubtedly many carriers enter the United States and engage in the raising of small fruits and vegetable. In many oriental countries, on account of the necessity for intensive cultivation, the use of human excreta as fertilizer has been a common procedure. Employment of this method of fertilization in the state of California has been a sufficiently common practice to prompt the California State Board

of Health¹⁴ to pass resolutions prohibiting its continuance in the production of vegetables, berries and low-growing fruits. Even after effective measures against this procedure have been taken, there still remains the danger of contamination of garden truck through cultivation and handling by carrier of *E. dysenteriae*.

The employment of such carriers in connection with the preparation of food in private homes or in public eating houses might also make possible the spread of amebiasis. Typhoid fever and bacillary dysenteries are known to be spread in this way and the conditions are equally favorable for the spread of amebic dysentery.

In addition to the factors mentioned, one other has recently been brought to attention by the work of Wenyon and O'Connor.¹² House flies fed by them on infected feces were found to pass living, apparently unchanged *E. dysenteriae* cysts during a period beginning 5 minutes after ingestion and continuing as long as 42 hours. They also examined between 200 and 300 wild flies and found about 8% carrying cysts of human intestinal protozoa, beside eggs of flukes, tapeworms and nematodes, commonly parasitic in the intestine of man. Over 3% carried cysts of *E. dysenteriae*. These flies were taken in a city where sanitary conditions were anything but good.

California is unique in having certain conditions and combinations of conditions favorable to the spread of amebic dysentery. Within the state are large numbers of people from countries where the disease is prevalent, many of whom are undoubtedly carriers. A large majority of these engage in agriculture, in the form of truck gardening, or become cooks and domestic servants. The state also has extensive irrigated areas that are seldom, if ever, visited by a considerable period of freezing temperatures.

California is also uniquely situated with respect to the introduction of the disease. Yearly there have come in a large number of immigrants, the greater part of whom have settled in the state. These immigrants have come principally from Japan, China, and India, where intestinal protozoa are common. Travel to and from the Hawaiian and Philippine Islands takes place through the ports of California. It is also likely that a larger percentage of Californians than of citizens of any other state visit these islands. This affords an important source for introduction of amebic dysentery as is indicated by numbers 3, 4, 5, 6, 10 and 12 of table 1. Into the southern part of the state there is

¹⁴ California State Board of Health, Monthly Bull., 1918, 13, pp. 451 and 461.

a large immigration of Mexican laborers. They no longer settle solely within the southern counties, but are becoming generally distributed throughout the state. There is also considerable travel to and from Mexico and the Central American States, due to mining interests and the operation of the Panama Canal. Introduction from these regions is undoubtedly frequent, as illustrated by cases 8, 13 and 17 of table 1.

The opportunities for the introduction and spread of *E. dysenteriae* in California are such that this parasite is probably much more prevalent than records show, and may become a serious menace not only to the state, but to the whole country.

OBSERVATIONS ON STREPTOLYSIN

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As is well known, certain streptococci produce hemolysin (streptolysin), concerning the nature and mode of production of which, as well as its relation to virulence, there are differences of opinion.

Besredka¹ found that only those streptococci which were virulent for rabbits produced hemolysin, and he recommended that in the study of the hemolysin, cultures should be made directly from the heart blood of rabbits that are dead from streptococcus infection. Ruediger² found only a slight hemolytic property in the filtrates of streptococcus culture in heated rabbit serum after 24 hours. Schlesinger³ was unable to obtain any production of hemolysin by streptococci that did not originally produce hemolysis, even when they had become virulent from passage through animals. Of 16 strains of streptococci Kerner⁴ found that 11 produced hemolysis; he also noted that the production was more marked in cultures made directly from an animal than when artificial cultivation had been carried on for some time. M'Leod⁵ asserts that there is a close association between the virulence and hemolytic power of certain streptococci. Landsteiner⁶ was unable to obtain hemolysin except from a few streptococci and then only after they had been passed through animals. Sachs⁷ does not agree with these investigators. His results indicate that there was no constant difference in the production of hemolysin by strains cultivated for some time and strains freshly isolated. He was unable to secure any filtrable hemolysin. Of the corpuscles of man, rabbit, dog, goat, sheep, beef, swine, guinea-pig, chicken and horse, those of man and rabbit were found the least resistant by Ruediger. He worked with a streptolysin which completely laked one drop of a thick suspension of washed corpuscles in 1½ hours. The corpuscles of chicken, beef and horse were somewhat more resistant than the other corpuscles mentioned, but as they could be made more susceptible by washing 5 times in salt solution, there seemed to be something in the serum of these animals that protect the corpuscles against the streptolysin, and he made careful tests of the antilysin. Besredka says that lysin produced by streptococci in human serum lyses human, rabbit, guinea-pig, goat and sheep corpuscles, but not chicken and goose; streptolysin produced in goat serum acted on human, rabbit and guinea-pig corpuscles, but not on goat, sheep, chicken and goose corpuscles, and he concludes that streptolysin and probably also the product of other bacteria may be different if produced in different mediums. Ruediger was unable to verify these results, streptolysin produced in goat serum, as well as that produced in human or

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¹ Ann. d. l'Inst. Pasteur, 1901, 15, p. 880.

² Jour. Am. Med. Assn., 1905, 44, p. 198.

³ Ztschr. f. Hyg. u. Infektionskr., 1903, 44, p. 428.

⁴ Centralbl. f. Bakteriologie, I, O., 1905, 38, p. 223.

⁵ Jour. Path. and Bacteriol., 1912, 16, p. 321.

⁶ Centralbl. f. Bakteriologie, I, Ref., 1906, 42, p. 785.

⁷ Ztschr. f. Hyg. u. Infektionskr., 1909, 63, p. 463.

rabbit serum laking goat, sheep and chicken corpuscles; he found that the streptolysin produced in rabbit serum acted more quickly than the others. Braun⁸ found that on blood agar streptococci may produce filtrable lysin, sometimes in large quantity, after a growth of from 8-10 hours. He found also that the lysin may be very sensitive and lose in strength after a few hours at 37 C.; the filtrate of the culture fluid was toxic for rabbits after cultivation for 10 hours but not for mice or guinea-pigs. In his experiments the most sensitive corpuscles were rabbit, human and guinea-pig; normal rabbit, guinea-pig, horse and human serum contained antistreptolysin. Kerner⁴ observed that the corpuscles of the dog were more sensitive to streptolysin than those of man or frog.

The question arises as to what relation there may be between the production of streptolysin and the virulence of streptococci. Do the more virulent streptococci produce more lysin than the less virulent? If so, is this true under all conditions? Another question is the cause of the varying sensitiveness of corpuscles of different animals to the action of streptolysin. In order to throw light if possible on some of these questions, experiments have been made, the results of which I present.

THE RELATION OF THE PRODUCTION OF STREPTOLYSIN TO THE MEDIUM, TIME AND ACID FORMATION

In my experiments I have used a streptococcus from an abscess of the frontal sinus and cultivated artificially for about a year. It is hemolytic on blood agar and forms long chains in dextrose broth. After trial I found that animal serum or broth mixed with serum is the best medium for the production of streptolysin. Plain broth and rabbit serum in different proportions, the serum being first heated at 56 C. for half an hour and incubated at 37 C. for 24 hours to test its sterility, was inoculated with streptococci, and the development of lysin studied with reference to time and acidity. The corpuscles used were washed at least 3 times in 0.9% salt solution and suspended in such solution. The serum broth cultures when filtered through Maassen filters gave a light yellow, clear and absolutely sterile filtrate. The mixture of culture fluid or filtrate and blood corpuscles was placed at 37 C. for 2 hours, with occasional shaking, and then in the icebox, the result being read after 15 hours. In order to determine the acidity, 10 c c of the medium was added to 90 c c of distilled water and titrated with phenolphthalein and N/10 NaOH. The tests of the streptolysin have been made as shown in table 1, which gives the proportions of serum and broth as 1:2, 1:1, 2:1, 4:1. These are the best mixtures

for the production of streptolysin. We see that in 5 hours the production was rather scanty, but after 10 hours a sudden increase occurred which continued for some hours, but after 48 and 72 hours the production gradually decreased. Braun also found the lysin production marked after from 8-10 hours' cultivation, and he emphasizes that the lysin is sensitive and that at 37 C. the lytic power may be destroyed in 6 hours. Hellens⁹ seems to have reached the same result. Sekiguchi,¹⁰ testing the relation between lysin and acid production, found that the production of lysin begins and ends earlier than the production of acid, lysin reaching its climax in from 15-18 hours, the acid in from 24-48 hours.

NATURE OF STREPTOLYSIN

Does the lysin in cultures of certain streptococci in serum broth lake different blood corpuscles in the same degree? If not, why? Is there any difference in the lysin production of virulent and nonvirulent streptococci? As shown in table 2, the streptococcus as isolated (1) and without having been passed through any animals, acts in about the same way on the corpuscles of different animals except that guinea-pig corpuscles seem to be a little more resistant; after passing the streptococcus through rabbits (2) its lytic power for various corpuscles is stronger, more particularly for rabbit corpuscles; when the strain that had been passed through rabbits passed through mice (3), the lytic power for mouse corpuscles is noticeably increased in a specific manner; when now the streptococcus is passed through a guinea-pig (4), the lytic action on guinea-pig corpuscles is stronger than for other corpuscles. The same results were obtained with the filtrates of the different cultures, although not in such marked degree. The results show that the production of lysin by this streptococcus increases as virulence is increased by passage through animals, and that the nature of the lysin changes according to the animal, being strongest for the corpuscles of the animal through which the strain in question has been passed last.

HEMOLYSIS BY CULTURE CENTRIFUGATES, STREPTOCOCCUS EMULSION, AND CULTURE FILTRATES

The results of the previous work on the filtrability of streptolysin have been divergent. I have found that a filtrable streptolysin may be obtained, but its lytic power is less than that of the unfiltered culture

⁹ Centralbl. f. Bakteriol., I. O., 1913, 68, p. 602.

¹⁰ Jour. Infect. Dis., 1917, 21, p. 475.

TABLE 1
PRODUCTION OF STREPTOLYSIN IN BROTH WITH VARYING PROPORTIONS OF SERUM, WITH REFERENCE TO TIME AND ACID FORMATION

Quantity of Culture Fluid	Salt Solution 0.3%	5% Suspension Rabbit Blood	Lysis																			
			Serum 1, Broth 2					Serum 1, Broth 1					Serum 2, Broth 1					Serum 4, Broth 1				
			Ages of Cultures in Hours																			
			5	10	24	48	72	5	10	24	48	72	5	10	24	48	72	5	10	24	48	72
1.0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
0.5	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
0.25	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
0.125	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
0.05	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
0.025	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
0.0125	0	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++		
Control	1.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
Degree of acid			0.9	0.9	0.9	1.1	1.2	0.9	0.95	1.1	1.1	1.2	0.8	0.9	0.9	1.3	0.9	0.85	0.9	1.2		

++++ = complete hemolysis.
 +++ = strong hemolysis.
 ++ = moderate hemolysis.
 + = trace of hemolysis.
 0 = no hemolysis.

The streptococcus used in this experiment was obtained originally from the frontal sinus and the cultures used here were of a strain that had been passed through five rabbits and then through nine mice; the culture fluid in this case was not filtered.

TABLE 2
LYSIS BY UNFILTERED AND FILTERED CULTURE FLUID OF DESCENDANTS (1, 2, 3, 4) OF THE SAME STREPTOCOCCUS BUT
SUBJECTED TO DIFFERENT TREATMENT

[illegible]

fluid (table 3). A streptococcus culture in serum broth 1:1 was incubated for 10 hours; the particular strain had been passed through 5 rabbits and 18 mice. The culture was centrifugated at a rapid rate for about 1 hour, the sediment washed with 0.9% salt solution 3 times and resuspended in an amount of salt solution two-thirds that of the original culture. At the same time, the culture fluid was filtered through a Maasen filter. Table 3 shows that the filtrate and the centrifugate were lytic, the filtrate being less powerful, but the suspension of washed streptococci was without any lytic action.

TABLE 3
COMPARISON OF HEMOLYSIS BY CULTURE FILTRATE, CULTURE CENTRIFUGATE AND STREPTOCOCCUS SUSPENSION

Quantity of Lytic Fluid	Salt Solution 0.9%	5% Suspension of Corpuscles	Lysis		
			Filtrate	Centrifugate	Streptococcus Suspension
1.0	...	1.0	++++	++++	±
0.75	0.25	1.0	+++	++++	0
0.5	0.5	1.0	+++	+++	0
0.25	0.75	1.0	++	++	0
0.125	0.875	1.0	+	+	0
0.05	0.95	1.0	±	+	0
0.025	0.975	1.0	0	+	0
Control	1.0	1.0	0	0	0

RELATION OF SPECIFIC VIRULENCE AND PRODUCTION OF STREPTOLYSIN

As stated, a streptococcus after being passed through a given animal produces a lysin which seems to be somewhat specific for the corpuscles of that animal. The strain passed through 5 rabbits, 15 mice, and then through 7 guinea-pigs, gave results indicating that as the virulence for a particular animal increases, the power of specific lysin production also increases. I have found that this power may be retained, although with gradual weakening, over a period of 42 days through 7 generations on goat blood agar. In other words, when a streptococcus has acquired the power of producing lysin for a particular kind of corpuscle, that power may be retained for some little time. My results in general show, however, that unless the streptococcus is passed through animals at short intervals it does not produce much lysin. If cultivated directly from the heart blood of an animal that has died in consequence of its injection, the streptococcus is virulent, but if grown through several generations on blood agar the viru-

TABLE 4
HEMOLYTIC ACTION OF FILTRATE OF CULTURES OF STREPTOCOCCI AFTER PASSAGES THROUGH GUINEA-PIGS

[illegible]

lence is reduced while the production of streptolysin is not affected so much, as shown in table 5. After a longer time, however, of cultivation on blood agar, say 6-7 generations over a period of 42 days, the lysin production becomes reduced. The fact remains that virulence for animals and lysin production do not run strictly parallel. A non-virulent strain grown on artificial medium for a long time, however, is hemolytic still on blood agar.

TABLE 5
RELATION BETWEEN VIRULENCE AND PRODUCTION OF HEMOLYSIN

Generations of Streptococcus on Blood Agar	Virulence as Indicated by Fatal Dose for Mouse of 24-Hour Culture in Serum Broth	Production of Hemolysin. The lytic strength is indicated by the percentage of the corpuscle suspension, 1 c c of which was laked completely by 1 c c of the fluid of a 24-hour serum-broth culture	
		Mouse Corpuscles	Guinea-Pig Corpuscles
Original serum broth culture from blood of mouse....	0.00001 c c	5%	1%
First generation on blood agar.....	0.001 c c	5%	1%
Second generation on blood agar.....	5%	1%
Third generation on blood agar.....	5%	1%
Fourth generation on blood agar.....	0.05 c c	5%	1%
Fifth generation on blood agar.....	5%	1%
Sixth generation on blood agar.....	2.5%	1%
Seventh generation on blood agar.....	2.5%	1%

THE EFFECT OF LYSIN ON CORPUSCLES AND QUANTITATIVE RELATIONS

It has been noted that a certain relationship must be secured between the lysin and the quantity of corpuscles in order to obtain the maximum lytic affect. I have tested the action of a fixed quantity of lysin on different corpuscles, and the results here also show that when a streptococcus is passed through a number of animals the lysin produced by that streptococcus is most active for the corpuscles of the last animal through which it passed.

TOXIC ACTION OF STREPTOLYSIN

According to Ruediger² and Braun, streptolysin is toxic for rabbits. I have found that 3-5 c c of lysin injected intravenously cause an attack of diarrhea with emaciation in rabbits; 1-2 c c injected into the abdominal cavity in mice would cause diarrhea lasting for several days, the animals in both cases recovering. Small quantities were without any apparent effect.

ABSORPTION EXPERIMENTS WITH STREPTOLYSIN

If the lysin produced by a streptococcus that has been passed through a certain animal has a specific action on the corpuscles of that animal, absorption tests with different corpuscles should reveal a similar specific relationship. Ruediger, Schlesinger and Hellens found that streptolysin combined with corpuscles at low temperature without causing lysis. I have made the following experiments bearing on this point:

To 20 drops of a thick suspension of mouse corpuscles were added 10 c c of streptolysin, the mixture kept at 0 C. for 5 hours and then centrifugated. The color of the fluid showed a trace of red. Similarly, another mixture was made with washed mouse corpuscles, and after centrifugation the corpuscles washed once more with 0.9% salt solution. On the addition of one drop of thick corpuscles suspension to 4 c c of the fluid of the first mixture, and after incubation for 2 hours and standing in the icebox for 15 hours, only a trace of hemolysis resulted. If one drop of the sediment of the second mixture was added to 4 c c of 0.9% salt solution, incubated for 2 hours and placed in the ice chest for 15 hours, marked hemolysis resulted. Untreated streptolysin caused complete lysis of mouse corpuscles in the control tests. This result shows that at low temperature there occurs a union between the lysin and the corpuscles, but no lysis. On injection of the clear fluid and the corpuscles in animals, the following results were obtained: In mice 1-2 c c of the clear fluid injected into the abdomen caused the same symptoms, with diarrhea, as the injection of filtrate of streptococcus culture. On the other hand, the injection of mice with 2-4 drops of mouse corpuscles treated with streptolysin, washed and then suspended in 2 c c of 0.9% salt solution, caused no symptom.

As these results indicate that the animals injected with fluid free from lysin developed the same symptoms as those injected with the untreated fluid, while the animals injected with the sediment remained well, some doubt is cast on the conclusion that the toxicity of streptococcus filtrates for certain animals is due to the lysin in such filtrates.

SUMMARY

The hemolysin production by streptococci is most successful in cultures in plain broth with equal or larger quantities of serum.

In such cultures the production of hemolysin is greatest after incubation at 37 C. for 10-24 hours; before 5 hours and after 48-72 hours only little lysin is produced.

There is a certain relation between the virulence of streptococci and the production of hemolysin. The production increases on successive animal passages as compared with the production in cultures of the same strain maintained on artificial medium.

The hemolysin produced by streptococci is filtrable, but it loses somewhat in strength on filtration.

Streptococci isolated from human beings and grown on artificial mediums for some time produce hemolysin that has nearly the same effect on the corpuscles of different animals, but when a streptococcus is passed through a number of animals of the same kind, its hemolysin production is increased, and this increase is most marked for the corpuscles of the animals through which the streptococcus has been passed. It seems as if the nature of the hemolysin depends to some extent on the host. If a streptococcus strain is passed through two species of animals, the corresponding hemolysin will be more active for the corpuscles of the species that harbored the streptococcus last.

Streptolysin unites with the corpuscles which it lyses, and streptococcus filtrates may remain toxic for mice after removal of the streptolysin by absorption with corpuscles.

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